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TOXICITY AND BIOCOMPATIBILITY OF NANOPARTICLES, AND STUDIES ON OXIDATIVE STRESS AND DNA DAMAGE

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Cover:
Upper photo: Sea buckthorn in the Swedish archipelago, photo by Eva Larsson.
Bottom left: Image of comets obtained in the comet assay to assess DNA damage in single cells.
Bottom right: TEM image of MCF-7 cells exposed to mesoporous silica nanoparticles, photo by Kjell Hultenby.

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To my Family and Friends

ABSTRACT

Oxidative stress is associated with several diseases, either as a cause or a consequence. Chronic kidney disease (CKD) is one example of a disease in which elevated levels of oxidative stress are frequently reported. Oxidative stress, inflammation and malnutrition are risk factors that contribute to an increased risk for cardiovascular disease and a higher morbidity and mortality in CKD patients. In addition, oral complaints such as periodontitis and mouth dryness are recurrently reported. Furthermore, oxidative stress can also be induced by exogenous sources such as toxic agents in our environment. Nanoparticles, which are increasingly used in the society, can potentially cause adverse health effects with oxidative stress as a proposed underlying toxic mechanism. Yet, nanoparticles offer tremendous possibilities for the society, not least within biomedical applications.

The aim of Study I in this thesis was to investigate the effects of kidney disease on DNA damage and oxidative stress in the salivary glands. The comet assay was used for the analysis and the results showed that the DNA damage in predialysis patients (CKD patients not yet on dialysis) was higher compared to controls as well as CKD patients on dialysis. The dialysis patients showed lower levels of DNA damage compared to the controls. There were no differences between the groups regarding the oxidative DNA damage. The inflammation and uremic markers were elevated in all CKD patients compared to the controls. The results suggest that the DNA in salivary glands are affected differently compared to in circulating blood cells that have been studied in previous studies in CKD patients, potentially due to upregulated DNA repair and antioxidative mechanisms in the peripheral tissue.

The aim of Study II was to examine health effects of dietary supplementation with an extract of sea buckthorn rich in antioxidants and fatty acids. The patient group was dialysis patients and the main outcomes were DNA damage and oxidative stress in the salivary glands, as well as saliva production. No significant effects on DNA damage, oxidative DNA damage or saliva production were observed in this crossover intervention study (2 × 8 weeks).

A further aim of the thesis was to investigate the toxicity of nanoparticles *in vitro.* A wide range of nano- and microsized particles was screened for cytotoxicity. Cu- and Zn-based (Cu, CuO, CuZn, Zn, ZnO) nanoparticles were found to be particularly cytotoxic. The Cu-based particles were cytotoxic in a size-dependent manner. Furthermore, the toxicity was found to be dependent on the type of cell investigated.

In Study III the aim was to elucidate the toxic mechanisms of Cu-based (Cu and CuO) nanoparticles. The studies were performed in a leukemic cell line, and the results showed that the Cu nanoparticles were most cytotoxic, followed by $CuCl_2$ and lastly CuO nanoparticles. The Cu nanoparticles induced high levels of oxidation in an acellular method, as well as slightly increased levels of intracellular reactive oxygen species (ROS) and oxidative DNA damage in the cells. CuO nanoparticles did not induce acellular ROS, and the induction of intracellular ROS and DNA damage was

limited. Differences in metal release processes may explain the differences in toxicity modes between Cu and CuO nanoparticles.

In Study IV, the application of nonporous and mesoporous amine-modified silica nanoparticles as plasmid delivery vectors was explored. Both of the silica particles were found to be biocompatible in the human breast carcinoma cell line that was studied. Nonporous particles were more efficient in the delivery of the plasmids. Addition of serum in the cell medium increased the delivery efficiency as well as restricted the toxicity.

In conclusion, the studies in this thesis indicate that the DNA in the oral tissue is less affected by kidney disease and the dialysis treatment compared to circulating blood cells. Dietary supplementation with a berry extract containing fatty acids and antioxidants appears to not affect the levels of DNA damage and oxidative damage in the oral tissue. Moreover, certain nanoparticles are toxic, and the toxicity is dependent on several factors including the chemical composition, particle size and dissolution. Concurrently, some nanoparticles, including silica nanoparticles, offer exciting possibilities to be used as platforms for biomedical applications. Taken together, further investigations on both toxicity and application of nanomaterials are required to avoid negative health effects as well as to embrace their beneficial use.

LIST OF PUBLICATIONS

I. Ersson C., Thorman R., **Rodhe Y**., Möller L. and Hylander B. **DNA damage in salivary gland tissue in patients with chronic kidney disease, measured by the comet assay.** *Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011; 112(2):209-15.*

II. **Rodhe Y**., Woodhill T., Thorman R., Möller L. and Hylander B. **The effect of sea buckthorn supplement on oral health, inflammation, and DNA damage in hemodialysis patients: a double-blinded, randomized crossover study.** *J Ren Nutr 2013; 23(3):172-9.*

III. **Rodhe Y**., Skoglund S., Odnevall Wallinder I., Potácová Z. and Möller L. **Copper-based nanoparticles induce high toxicity in leukemic HL60 cells.** *Submitted to Toxicology in vitro.*

IV. Shi J., **Rodhe Y**., Ersson C., Geny S., Ye F., Muhammed M., Smith E. and Möller L. **Amine-modified silica nanoparticles as non-viral vectors for the delivery of plasmid DNA.** *Manuscript*

Additional paper not included in the thesis

Rodhe Y., Skoglund S., Odnevall Wallinder I., Potácová Z. and Möller L. **Assessment of toxic mechanisms of zinc and zinc oxide nanoparticles in HL60 cells and evaluation of cell-type specific cytotoxicity.** *Manuscript*

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

A	Adenine
AAS	Atomic absorption spectroscopy
AP	Apurinic and Apyrimidinic
AP-1	Activator protein-1
C	Cytosine
CKD	Chronic kidney disease
CRP	C-reactive protein
DCF	2',7'-dichlorofluorescein
DCFH-DA	2',7'-dichlorofluorescein-diacetate
dG	2'deoxyguanosine
DLS	Dynamic light scattering
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
EPR	Enhanced permeability and retention
FACS	Fluorescence-activated cell sorting
FPG	Formamido pyrimidine DNA glycosylase
G	Guanine
GST	Glutathione S-transferase
HO-1	Haem oxygenase 1
hOgg1	Human 8-oxoguanine glycosylase 1
IL	Interleukin
LPC	Leukocyte-particle concentration
MAPK	Mitogen-activated protein kinase
MRI	Magnetic resonance imaging
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MWCNT	Multi-walled carbon nanotubes
NF-κB	Nuclear factor-κB
Nrf-2	Nuclear factor erythroid 2-related factor 2
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCCS	Photon cross-correlation spectroscopy
PEG	Polytehylene glycol

PEI	Polyethyleneimine
PI	Propidium iodide
PS	Phosphatidylserine
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI	Roswell park memorial institute medium
SBO	Sea buckthorn oil
siRNA	Small interfering RNA
SOD	Superoxide dismutase
T	Thymine
TEM	Transmission electron microscopy
TMRM	Tetramethyl rhodamine methyl ester
TNF-α	Tumour necrosis factor alpha
UNG	Uracil-DNA glycosylase
8-oxodG	8-oxo-7,8-dihydro-2'deoxyguanosine
8-oxoG	8-oxo-7,8-dihydro-guanine

1 INTRODUCTION

Our health can be affected by factors in the environment, life style and dietary habits, as well as by disease. Exposure to toxic agents present in the air, soil, food and water can damage our cells and lead to disease development, further affecting our life span and life-quality. Some toxic agents are linked to the generation of reactive oxygen species (ROS), which have the potential to damage cellular molecules. ROS are also continuously formed during normal cell metabolism. Cells and organisms have therefore evolved an extensive antioxidative system. Oxidative stress, a condition of an imbalance between oxidative and antioxidative processes, can result in damage to the DNA, proteins and lipids in cells. Cancer, cardiovascular disease, diabetes, kidney disease, Parkinson's and Alzheimer's disease are all examples of diseases associated with oxidative stress, either as a cause or as a consequence of the disease[1]. In agreement with the free radical theory of ageing, oxidative stress is also considered to play an important role in normal ageing processes[2].

The antioxidative system includes both endogenous antioxidants as well as antioxidants derived from diet. As some vitamins and other micronutrients have antioxidative properties, there is a great interest in dietary supplementation containing these substances, with the aim to improve the health and to prevent disease and ageing. The effects of these supplements are however unclear. Studies on dietary supplementation have not consistently shown beneficial health effects; several studies show lack of effects and some studies even show harmful effects[3].

The first part of this thesis is based on two human studies with chronic kidney disease (CKD) patients (Study I and Study II). CKD is a disease with a progressive loss of renal function, resulting in an augment of uremic toxins in the blood. Renal disease is associated with elevated levels of oxidative stress and inflammation, and increased risk for cardiovascular disease[4]. In addition, CKD patients also often report impaired oral health[5]. CKD is an increasing worldwide public health problem, with a global prevalence of 8–16%[6].

The aim of Study I was to assess the levels of DNA damage and oxidative stress in the salivary glands, as well as the potential correlation with saliva production, inflammation and uremic markers in CKD patients. The aim of Study II was to evaluate the effects of dietary supplementation with a sea buckthorn extract on the levels of DNA damage, oxidative stress, saliva production, inflammation and uremic markers in CKD patients. Sea buckthorn has been attributed with beneficial health effects including antioxidative, antiproliferative and anti-inflammatory effects, as well as improved function of mucous membranes[7-9]. Thus, it was hypothesised that the sea buckthorn extract could improve oral health, potentially by decreasing DNA damage and oxidative DNA damage in the salivary glands, as well as improve the saliva production.

Study I and Study II were previously summarised in the licentiate thesis "Assessment of DNA damage, oxidative stress and inflammation in chronic kidney disease patients – and a clinical study of a dietary supplement"[10]. While the main literature

review and the results and discussion on chronic kidney disease, dietary supplementation and sea buckthorn originate from the licentiate thesis, an updated version is presented in this thesis.

Exposure to nanomaterials is an environmental factor that could have impact on our health. The rapid development of nanotechnology enables engineering of nanomaterials with unique properties that make them attractive for usage within electronics, medicine, inks and cosmetics among other areas. The increased production and spread in the society can potentially increase the human exposure to nanomaterials, e.g. from the air, at working places or from consumer products. The exposure to nanomaterials may pose increased health risks as the small size of nanomaterials can implicate alterations in the interactions with biological systems, including the exposure and translocation routes, cellular uptake and toxic mechanisms[11,12]. To ensure a safe development of nanoproducts and nanomedicine, and to protect humans and the environment against adverse effects, there is a great need for a deeper understanding on how nanoparticles interact with biological systems.

The second part of this thesis is based on two *in vitro* studies on interactions of nanoparticles with human cells. The aim was to identify particularly toxic particles, to elucidate the toxic mechanisms of Cu-based (Cu and CuO) nanoparticles, as well as to investigate the biocompatibility and potential use of silica nanoparticles for gene delivery. A range of particles was screened for cytotoxicity, while cell-type specific cytotoxicity was investigated for a selection of the particles. The particularly toxic Cu-based nanoparticles were selected for further studies on toxic mechanisms in Study III. In Study IV, the possibility to use silica nanoparticles for gene delivery was explored. Their biocompatibility as well as their efficiency in delivering plasmid DNA in a breast carcinoma cell line was investigated.

As oxidative stress is involved in CKD as well as in the toxicity of nanoparticles, the next sections will therefore introduce oxidative stress, DNA damage, CKD, sea buckthorn, nanotechnology and nanotoxicology.

2 OXIDATIVE STRESS

All aerobic organisms require, as the name indicates, oxygen (O_2) for an efficient energy production. Energy is produced through the electron transport chain that takes place in the mitochondria in eukaryotic cells. Oxygen from the air is in humans transported via the lungs and blood to tissues and cells by a protein called haemoglobin. This transport is mediated by the haem groups of the protein, where the Fe^{2+} is oxidised to Fe^{3+} upon oxygen binding. In the cell, the electron donors NADH and $FADH_2$ deriving from metabolic pathways, are used for the step-wise reduction of O_2 that forms H_2O as the final end product, as seen in **Figure 1.** These electron transfers build up a proton gradient across the inner mitochondrial membrane, which can generate adenosine-5'-triphosphate (ATP), the energy source of the cell. The redox reactions taking place in the mitochondria are tightly controlled by specific enzymes. Despite the control, electrons are occasionally slipping which can lead to the formation of ROS. The mitochondria are considered to be the major endogenous source of ROS[13]. Moreover, ROS can also be produced by the cell as a part of its pathogen defence as well as for cell signalling. In addition to the endogenous sources of ROS, environmental factors including radiation or exposure to toxic compounds such as tobacco smoke, diesel exhaust, ozone or nanoparticles, can induce the formation of ROS in cells.

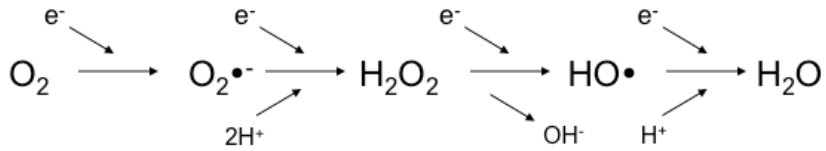

Figure 1. Electron transfers during the step-wise reduction of O_2 to H_2O taking place in the mitochondria.

The term ROS includes both radicals, i.e. species with one or more unpaired electrons in their atomic or molecular orbitals, and non-radical oxygen species. Examples of ROS that are formed in the cell are superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (HO•). Some of their cellular origin and defence are listed in **Table 1.** Molecular oxygen at ground state (O_2) can be classified as a free radical with two unpaired electrons (i.e. a diradical) with parallel spins, but the reactivity is limited due to spin restrictions. Upon the addition of one electron, the superoxide anion $O_2^{\bullet -}$ is formed, which is a free radical with one unpaired electron that reacts poorly with biomolecules in aqueous solutions[1]. Nevertheless, $O_2^{\bullet -}$ can cause cellular damage by reacting with other species such as nitric oxide and form peroxynitrite, or it can oxidise Fe-S clusters in enzymes resulting in enzyme inactivation[14]. Phagocytic cells produce $O_2^{\bullet -}$ by the enzyme NADPH oxidase upon the activation by foreign substances. Dismutation of $O_2^{\bullet -}$ generates H_2O_2, which is a relatively weak oxidant. However, H_2O_2 can be cytotoxic and inactivate enzymes[15]. In addition, H_2O_2 is a potential source of the hydroxyl radical HO•, which is

generated upon the reaction with transition metals (e.g. Fe, Cu) in the Fenton reaction [1].

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO\bullet \qquad [1]$$

The hydroxyl radical is extremely reactive and it can oxidise any cellular macromolecule close to its formation site, inducing radical chain reactions[1]. The rate of reaction is often only limited to the rate of diffusion of the reactants.

Other central ROS are ozone (O_3), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl) and peroxyls (R-OO•). Reactive nitrogen species (RNS), including nitric oxide (NO•), nitrogen dioxide (NO_2•) and peroxynitrite ($ONOO^-$) are also important sources of oxidative stress in cells.

Table 1. Examples of reactive oxygen and nitrogen species, and their cellular origin and defence.

ROS/RNS	Origin	Defence
Superoxide ($O_2\bullet^-$)	Leakage from the electron transport chain, NADPH oxidase, xanthine oxidase	Superoxide dismutase
Hydrogen peroxide (H_2O_2)	Superoxide dismutase, NADPH oxidase	Catalase, glutathione, peroxidase, peroxiredoxin
Hydroxyl radical (OH•)	Fenton reactions	Sequestering of metal ions, e.g. transferrin and metallothionein
Nitric oxide (NO•)	Nitric oxide synthase	Glutathione

ROS are, as mentioned above, important for several redox reactions in the cells, in the immune system, for cell signalling, cell growth and differentiation. Concurrently, ROS also present a threat to the cell, as they can oxidise and damage cellular macromolecules. The intracellular environment is generally highly reduced. As a consequence of the aerobic respiration, aerobic cells are constantly exposed to ROS, and have therefore evolved an antioxidative system for counterbalance. The balance between the oxidative and the antioxidative processes is important for maintaining the appropriate redox state necessary for normal cell growth and proliferation, as depicted in **Figure 2.** The term *oxidative stress* was formulated by Helmut Sies in 1985, and it describes the situation when there is a disturbance in the balance between oxidants and antioxidants, with the former overwhelming the latter[16].

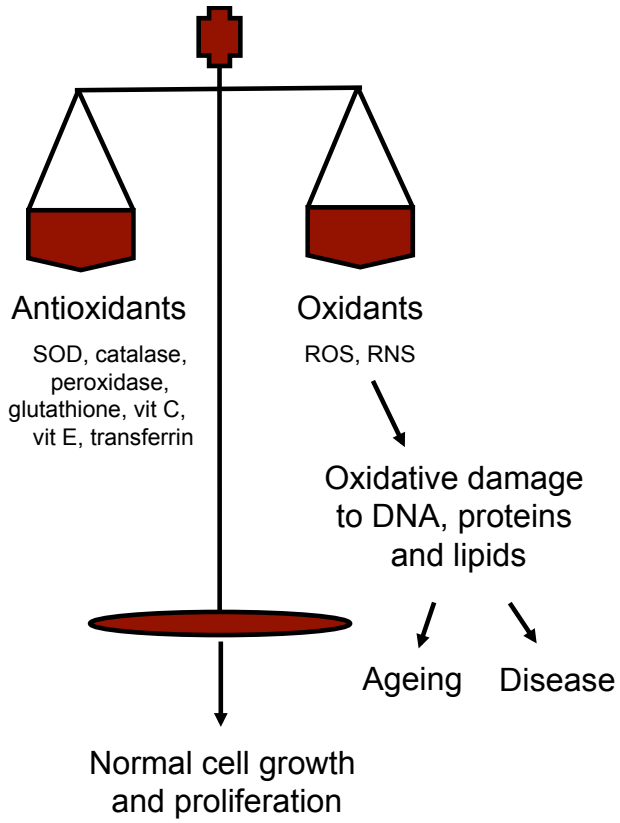

Figure 2. A balance between the oxidative and antioxidative processes in a cell is necessary for normal cell growth and proliferation. Excess levels of oxidants, or decreased levels of antioxidants, can cause damage to cellular macromolecules, potentially contributing to ageing processes or disease development.

The redox state (the relation between oxidative and reductive processes) in a biological system is dynamic; it can shift upon changes in the environment. To a certain extent, cells and organisms can make adaptations to increased oxidative processes by regulating the activity of specific genes and enzymes important for the antioxidative defence. Transcription factors such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) are regulated by the redox state in the cell[17]. Increased oxidative stress can promote signals for senescence and programmed cell death (apoptosis), and also lead to necrosis.

2.1 OXIDATIVE DAMAGE

ROS can cause oxidative damage to the DNA, proteins and lipids. This can lead to mutations in the genome or dysfunctions of the molecules, possibly leading to cancer, cell injury or cell death. This thesis is focused on damage to the DNA.

2.1.1 DNA damage

Our genetic information is stored in DNA, which is a molecule that is built up by four types of subunits; the nucleotides. Each nucleotide is composed of a five-carbon sugar, a phosphate group and one of the four bases adenine (A), cytosine (C), guanine (G) or thymine (T). Base pairing with hydrogen bonds between G and C, and T and

A connect two sugar-phosphate backbones, forming the double-stranded DNA molecule, depicted in **Figure 3**.

Figure 3. DNA molecule illustrated with PyMOL[18], courtesy Dr Arzu Uyar.

The DNA is, like other biomolecules, subject to damage and instability. The integrity of the genome is continuously compromised, both by spontaneous modifications and by modifications induced by exogenous factors[19,20]. The damage can be induced by oxidation, hydrolysis, alkylation and ionising radiation. The major forms of DNA alterations include single and double strand breaks, base lesions, cross-links, base misincorporations, bulky adducts and modifications of the sugar backbone. Cellular responses to DNA damage include the activation of DNA repair, changes in the gene transcription, triggering of apoptosis or cell cycle arrest[21]. Failure in the cellular response may lead to mutations, i.e. permanent changes in the DNA sequence, and subsequent loss of cellular regulation, potentially leading to cancer development.

All four bases and the sugar backbone of the DNA can be damaged by oxidation. Guanine has the lowest redox potential of the bases and is therefore the most readily oxidised base[22]. One of the most studied DNA oxidation product is 8-oxo-dG, which is formed after oxidation at the 8th position of dG (2´deoxy-guanosine)[23], as shown in **Figure 4**.

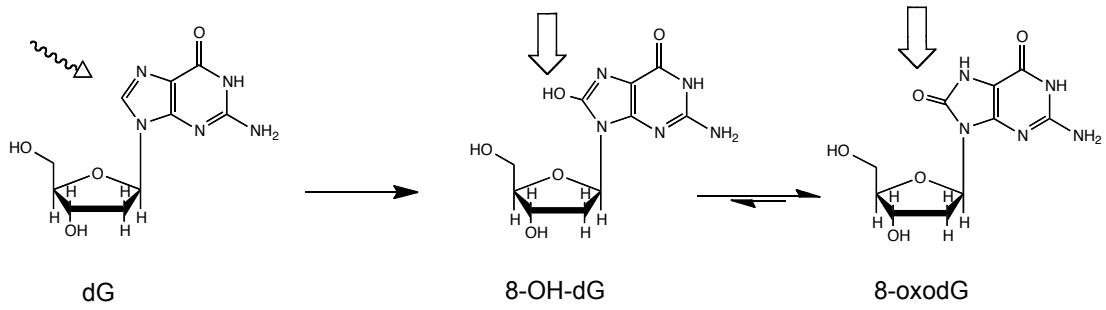

Figure 4. Oxidation of dG at the 8th position generates 8-OH-dG and 8-oxodG.

The oxidised form of guanine can assume *syn* conformation which allows base pairing with adenine, causing a transversion of G:C to T:A during the replication[24]. 8-oxodG is a common biomarker of oxidative stress and, due to its pro-mutagenic property, it is also a potential biomarker of carcinogenesis[25]. 8-oxodG and other DNA oxidation products can be detected and analysed by the comet assay, as described in section 7.1. As 8-oxodG is excreted in the urine, measurement of urinary levels of 8-oxodG is also a commonly used method to assess oxidative stress[26].

2.1.2 DNA repair

To counteract the constantly occurring DNA damaging processes, cells have evolved an extensive DNA repair system. The system consists of an ensemble of enzymes acting with different mechanisms and pathways depending on the type of damage. There are excision repair systems by which the damage is removed, mismatch repair systems that correct bases that have been misincorporated during the replication, and homologous and non-homologous recombination systems that repair double strand breaks.

Base excision repair is the most common repair process for damaged bases, including 8-oxoG. Damaged bases are recognised by glycosylases that remove the base via hydrolysis of the N-glycosyl bond between the sugar and the base, thus creating an abasic site. At this apurinic or apyrimidinic (AP) site, endonuclease or lyase activity creates a gap in the DNA backbone, which the DNA polymerase can fill with the appropriate nucleotide. The repair pathway is completed by ligase activity and sealing of the DNA. Human 8-oxoG DNA glycosylase (hOgg1) is an enzyme with both glycosylase and lyase activity, responsible for the removal of oxidised bases, including 8-oxoG[27]. Formamido pyrimidine DNA glycosylase (FPG) is the corresponding enzyme in *Escherichia coli,* and is often utilised to quantify oxidised DNA lesions in DNA analysis, including in the comet assay[28].

Bulky adducts, including pyrimidine dimers formed e.g. upon exposure to UV-light, are removed by nucleotide excision repair systems. This system recognises the damage and cuts the phosphodiester bonds via hydrolysis on both sides of the adduct, releasing an oligomer containing 24–32 nucleotides[29]. The gap in the DNA strand is subsequently filled and the nicks are ligated by DNA polymerase and DNA ligase.

2.2 ANTIOXIDATIVE DEFENCE

Regulation of the intracellular redox state is necessary for normal cellular function and protection of the biomolecules from damage. Cells have an extensive antioxidative system including mechanisms to prevent the formation of ROS, neutralise or remove ROS, as well as to repair the oxidative damage (e.g. the DNA repair, as described in section 2.1.2). The antioxidative system consists of a variety of enzymes, proteins and low molecular weight molecules such as glutathione and certain dietary antioxidants.

2.2.1 Antioxidant enzymes and proteins

Superoxide dismutase (SOD), catalase, glutathione peroxidase and peroxiredoxin are examples of antioxidant enzymes, which by catalysing the removal of ROS act as antioxidants. SOD catalyses the dismutation of $O_2^{\bullet -}$, which generates O_2 and H_2O_2. The human types of this enzyme include SOD1 (CuZnSOD present mostly in the cytosol but also in the intermembrane of the mitochondria), SOD2 (MnSOD present in the mitochondria) and SOD3 (extracellular glycosylated CuZnSOD)[30]. H_2O_2 that is formed during the dismutation of $O_2^{\bullet -}$, can be eliminated by catalases, which catalyse

the dismutation of H_2O_2 to H_2O and O_2. Since H_2O_2 is an important molecule for cell signalling, the elimination of H_2O_2 is well regulated. Glutathione peroxidase is a family of enzymes reducing H_2O_2 to H_2O (or lipid peroxides to alcohols) by selenocystein or cystein-dependent mechanisms leading to the oxidation of glutathione. The peroxiredoxin is an additional family of enzymes catalysing the reduction of H_2O_2. Glutathione is a thiol-containing tripeptide, functioning as a cellular redox buffer. Upon oxidation are two reduced glutathione (GSH) molecules linked via a disulfide bond, forming oxidised glutathione (GSSG). GSH can be regenerated by glutathione reductase. The ratio of GSH to GSSG is often used to express the redox state in a cell.

Several proteins act as antioxidants by limiting the presence of free metal ions, to prevent Fenton reactions and the formation of the reactive OH•. Since both copper and iron are essential for many proteins, the management and transport of these ions are tightly controlled in order to avoid leakage and pro-oxidant effects. Transferrin, lactoferrin, ferritin, metallothionein, caeruloplasmin and albumin are examples of proteins that are sequestering and managing the transport of iron and copper.

2.2.2 Dietary antioxidants

Dietary micronutrients include vitamins, carotenoids, trace elements, flavonoids and other phytochemicals. Vitamins are small organic molecules required in human diet, due to a lack of capacity to synthesise them in sufficient amounts[31]. There are 13 vitamins and they exert a broad range of functions including antioxidative properties. Vitamins A, C, and E are present in high levels in sea buckthorn berries and some of their physiological functions are presented below.

Carotenoids, a group of red and yellow coloured pigments, can be found in a variety of fruits and vegetables. They, including β-carotene, are important for humans as precursors of vitamin A. All carotenoids derive from a skeleton of 40 carbon atoms. Vitamin A is a designation of a group of fat-soluble compounds that includes retinol, retinaldehyde, retinoic acid and retinyl esters. They all have vitamin A-activity and are essential for several biological processes in humans, including the immune system, vision, cell growth and cell differentiation[1]. Vitamin A is ingested through the diet as retinyl esters from animal sources, or as provitamin A from plant sources[32]. The Swedish dietary recommendations for vitamin A compounds are 700–900 retinol equivalents (1 retinol equivalent = 1 μg retinol = 12 μg β-carotene) per day for healthy adults[33]. β-carotene is the most important provitamin A and is oxidatively cleaved in the intestine to achieve vitamin A-activity[1]. The carotenoids can act as antioxidants by quenching singlet oxygen, an important function in plant cells. In humans, the antioxidative effects of β-carotene are more uncertain and depend on the cellular environment. β-carotene can scavenge peroxyl radicals, forming an unstable β-carotene radical adduct that can further generate non-radical products[34]. However, if the oxidation products of β-carotene are not neutralised by other antioxidants, they can have pro-oxidative effects in the cell.

Ascorbic acid, well-known as vitamin C, is a water-soluble nutrient that has two ionisable OH-groups, and at physiological pH, the mono-anion ascorbate is the

predominant form[1]. While most animals and plants are able to synthesise ascorbate from glucose, humans lack an enzyme needed for this synthesis and thus need to ingest vitamin C through the diet. Ascorbate is a cofactor for several enzymes, including the enzymes needed for the proper biosynthesis of collagen, carnitine, norepinephrine, tyrosine metabolism and amidation of peptide hormones[35]. Lack of vitamin C can cause scurvy, a deficiency disease that leads to defect collagen[36]. Vitamin C is also important for iron absorption in the intestines and contribute to the regulation of iron homeostasis[37]. Plasma levels of vitamin C are in the range of 50–60 μM for healthy individuals, although the intracellular concentration can reach 1 mM in several cell types[38]. Ascorbate has a reducing ability and can act as a scavenger of ROS and RNS. The one-electron oxidation of ascorbate generates the ascorbyl radical, which can be further oxidised to dehydroascorbate. The antioxidant effect of ascorbate comes from replacing the damaging radicals by the less reactive ascorbyl radical. Ascorbate can also interact with the radical species from α-tocopherol and oxidised glutathione, regenerating the antioxidant molecules[36]. As vitamin C is a redox active molecule, it can also act as an oxidant. Pro-oxidative properties have been shown *in vitro*[39,40], however, the potential harm of pro-oxidative effects *in vivo* has been questioned[41]. Recommended daily intake of vitamin C for healthy adults in Sweden is 75 mg[33].

Eight fat-soluble tocopherol and tocotrienol derivatives belong to the vitamin E compounds. Their chemical structure includes a chromanol ring with one to three methyl groups and a side chain that contains either three double bonds (tocotrienols) or a phytyl chain (tocopherols). Both types have four isomers, i.e. α, β, γ and δ. The α-tocopherol is considered to be the most bioavailable vitamin E in humans[42], however, the importance of the other vitamin E compounds has been raised[43]. The vitamin E compounds are important for the cell membranes since they are able to scavenge peroxyl radicals, thereby inhibiting the free-radical chain reactions of lipid peroxidation. Tocopherols can also have pro-oxidative effects, by which the α-tocopherol radical may oxidise polyunsaturated fatty acids, which can lead to chain reactions of lipid peroxidation. Nevertheless, the rate constant of this reaction is much lower compared to the rate constant for the reaction between the peroxyl radical and the polyunsaturated fatty acids, and the importance of the α-tocopherol-mediated peroxidation *in vivo* is under debate[1]. Moreover, vitamin A can prevent such oxidation by recycling the α-tocopherol radical[44]. Tocopherols can also protect cellular membranes against singlet oxygen by quenching[44]. The Swedish dietary recommendations for vitamin E compounds are 8–10 mg α-tocopherol equivalents (1 α-tocopherol equivalent = 1 RRR-α-tocopherol) per day for healthy adults[33].

2.2.3 Dietary supplementation

There is epidemiological evidence that a diet rich in fruits and vegetables is inversely correlated with cancer, cardiovascular disease and mortality[45-48]. The positive health effects are often attributed to the vitamins and antioxidants, present in large amounts in fruits and vegetables. Therefore, much attention has been devoted to dietary supplements containing vitamins and antioxidants. However, human intervention studies have often failed to show beneficial health effects of vitamin supplements. Meta-analyses have even showed increased mortality associated with the intake of

β-carotene and potentially vitamin E and vitamin A[3]. The results highlight the complexity in the topic of antioxidant supplementation, suggesting that there are other important functions of the vitamins, and/or other components of fruits and vegetables, that play significant roles in disease prevention. The supplements studied have often contained single or a few vitamins at high concentrations, which can potentially disturb the balance in the redox state of cells and organisms. However, the studies also indicate that there may be certain groups that would benefit more from dietary supplementation, which potentially include poorly nourished groups, with low baseline nutritional status. For instance, in the Chinese Linxian study, with participants of poor nutritional health, the mortality decreased after supplementation with α-tocopherol, β-carotene and selenium[49]. Moreover, in the French SU.VI.MAX study it was found that supplementation with multivitamins and minerals lowered the incidence of cancer and mortality in men[50]. The beneficial effect was not seen among women and it was suggested to be due to the lower baseline levels of vitamin C and β-carotene in men compared to women. It must also be noted that some groups, such as smokers, are more susceptible for increased health risks of β-carotene supplementation, as observed in the CARET and the ATBC studies[51,52].

2.3 SEA BUCKTHORN

Extracts of fruits, vegetables and berries are applied as dietary supplements in an approach to mimic the natural content of the original specie. The sea buckthorn berry, seen in **Figure 5**, has recently raised more interest in the Western world, both as a part of the diet or dietary supplement, as well as in skincare products. Sea buckthorn has traditionally been used as a medicinal plant for several hundred years in China, Turkey and Russia. There are seven subspecies of sea buckthorn with different geographical origin; *Hippophae rhamnoides L.* is the most frequently grown species in Europe. The berry contains high levels of unsaturated fatty acids, vitamin C, vitamin E, carotenoids and phytochemicals including flavonoids and other phenolic compounds. The nutrient content varies among the different species and the geographic growth place as well as also among the different parts of the berry[53].

Figure 5. Sea buckthorn berries in the Swedish archipelago, photo by Eva Larsson.

The sea buckthorn berry has been attributed with antioxidative, anti-tumour, anti-proliferation and anti-inflammatory properties as well as immune response regulatory effects[7,54-58]. Recently, Widén *et al.*[59] demonstrated an antibacterial activity of sea buckthorn juice and suggested that it may reduce the risks for tooth decay and gingivitis. While most available studies are *in vitro* or *in vivo* animal studies using different extracts of the berry, only a few human studies on whole sea buckthorn berries or extracts have been published. Reported effects in humans include inhibition of induced platelet aggregation[60], reduction of serum levels of C-reactive protein (CRP)[8], metabolic effects[61] and an attenuated increase of tear film osmolarity in people suffering from dry eyes symptoms[9].

3 CHRONIC KIDNEY DISEASE

3.1 RENAL FAILURE

In CKD, the functional units of the kidneys, the nephrons, are progressively lost, which causes a decrease in renal function. This will lead to an augment of uremic symptoms including increased levels of urea and creatinine in the blood, anaemia and disturbances in the electrolyte and water balance. End-stage renal disease is managed by a protein-reduced diet, medication for hypertension and anaemia, correction of electrolytes, as well as renal replacement therapy including dialysis treatment or kidney transplantation. Dialysis treatment, performed to replace an impaired kidney function, is an artificial purification of the blood where waste products are removed and the balance between salts and fluids can be regulated. In haemodialysis, the blood is pumped out of the patient's body and filtered using a dialyzer before it returns to the patient. The dialyzer is designed with a semi-permeable membrane allowing passage of substances and fluids between the blood and the dialysate. In peritoneal dialysis, the exchange of substances and fluids is performed using the patient's peritoneal membrane inside the abdomen as a filter[62].

3.2 OXIDATIVE STRESS, INFLAMMATION AND ORAL HEALTH IN KIDNEY DISEASE

Kidney failure is associated with high levels of oxidative stress, inflammation and malnutrition. These factors are closely related and contribute to the higher risk for cardiovascular disease, atherosclerosis and cancer in CKD patients[63-66]. Elevated levels of oxidative stress are frequently reported in CKD patients[4,67-69]. Beyond the generation of ROS during normal cellular metabolism, potential sources of oxidative stress in CKD patients also include elevated systemic inflammation, incidence of diabetes, the dialysis treatment, reduced dietary intake of antioxidants and the accumulation of uremic toxins. Raised levels of inflammation markers, such as specific cytokines and acute-phase reactants, are often observed in CKD patients[4,67,68].

The dialysis treatment itself can also be a source of oxidative stress and inflammation. The contact between the blood and the dialysis membrane can trigger inflammatory reactions and alterations in the constitution of blood cells during dialysis. The activation of the complement system depends on the biocompatibility of the dialysis membrane. For instance, Memoli *et al.*[70] showed that peripheral blood mononuclear cells (PBMC) collected after cuprophane membrane-haemodialysis produced higher levels of interleukin (IL)-12 compared to polymethylmethacrylate membrane-haemodialysis, in both healthy controls and CKD patients. ROS can also be generated in the process of neutrophil activation during which the NADPH oxidase complex throughout the respiratory burst produces O_2^-. In addition, dialysate impurities such as endotoxins and bacterial cell wall fragments, can stimulate the activation of monocytes and the production of cytokines including IL-1 and TNFs[71].

The prevalence of anaemia, characterised by a decreased number of red blood cells, is high in CKD patients. Anaemia can be a consequence of several factors including a reduced production of erythropoietin (a hormone important for production of red blood cells), blood loss, lack of available folate and vitamin B_{12}, and iron deficiency. Impaired erythropoiesis is improved by erythropoiesis-stimulating-agents and supplementation with oral or intravenous iron. However, appropriate dosing of iron is complex, as an iron overload can contribute to the increased risk for cardiovascular disease in CKD patients[72]. Iron supplementation is a potential source of oxidative stress since free iron, not bound to iron-binding proteins such as transferrin, can participate in Fenton reactions generating hydroxyl radicals. Lipid peroxidation has been shown to increase shortly after intravenous iron infusion[73,74]. However, the significance of these pro-oxidative effects is uncertain and the benefits of iron supplementation upon iron repletion and anaemia are central[75]. Intravenous iron supplementation in appropriate doses and preparation is widely used and recommended[72,76].

Malnutrition and protein-energy wasting are considered as additional risk factors for the high mortality observed in CKD patients[77,78]. Dietary restrictions, such as reduced intake of proteins, potassium, sodium, phosphorus, calcium and excess fluid, may be required to diminish the complications of kidney failure and dialysis treatment. Alteration in the diet, in some cases with reduced intake of fruits and vegetables in order to evade hyperkalaemia and decrease levels of phosphorus and calcium, is a potential cause of antioxidant deficiency[79]. Water-soluble vitamins such as vitamin B and vitamin C are also lost during the dialysis treatment.

The oral health status is known to affect the general health of humans; inflammation and complaints in the oral cavity increase the systemic inflammation and contribute to atherosclerosis and cardiovascular disease development[80]. Poor oral health is common among CKD patients[81]. Several studies report impaired oral health status including periodontitis (tooth loss), xerostomia (mouth dryness), mucosal lesions, gingival enlargement, and changes in the saliva[5,82-84]. The impairments can be caused by medications, reduced oral care by the patients, increased levels of carbohydrates in the diet or by dysfunction of the immune defence caused by the uraemia and dialysis treatment[85]. Oxidative stress and damage to the salivary gland tissue have been suggested as underlying factors to oral complaints[86]. Appropriate function of the salivary glands is necessary for a good saliva production and oral health. The production of the saliva is managed by three pairs of major salivary glands and hundreds of minor salivary glands throughout the mouth. The inner lip area, as seen in **Figure 6**, is one of the locations with abundant number of minor salivary glands. The saliva is essential for maintaining a good oral health by protecting the oral tissue from infections and breakdown. Measurements of saliva constituent as well as saliva production, can provide information regarding salivary gland function and oral health status.

Figure 6. Several hundreds minor salivary glands are present throughout the oral cavity (located in lips, cheeks, hard and soft palate and the tongue), photo courtesy Amela Trbakovic, DDS.

4 TOXICITY AND BIOCOMPATIBILITY OF NANOPARTICLES

4.1 NANOTECHNOLOGY

Nanotechnology can be described as the technology in which objects that are in the size-range of nanometres are produced, engineered or utilised. The prefix *nano* refers to a factor of 10^{-9}, which means that 1 nanometre (nm) is equal to a billionth of a meter. Nanotechnology has developed rapidly during recent years and applications of nanomaterials can be found within several sectors in the society. The key feature of nanomaterials is their small size. The small size can yield unique physicochemical properties, different from larger-sized particles and bulk material of the same chemical composition. One key factor behind the difference is the large percentage of atoms localised on the surface of the particles, that is, the smaller-sized particles have a larger surface area-to-mass ratio[87]. In addition, the surface atoms have lower coordination number and are less stable than the bulk atoms. This is especially relevant for nanoparticles less than 30 nm in size, where it can influence the surface reactivity and melting point[88]. Another key factor is the impact of quantum effects, which can alter optical and magnetic properties, conductivity and catalytic activity of nanomaterials[89]. It should be noted that the impact of these factors varies with particle size within the nanoscale, and also depends on the chemical composition and crystalline structure of the particle. The possibility to tune these properties makes nanomaterials highly attractive for several applications.

Even though the anthropogenic sources of nanoparticles have increased in recent years, the presence of nano-sized materials is not new, as humans have been exposed to nanoparticles throughout the evolution. Examples of sources of naturally occurring nanoparticles are volcanic ash, sea spray and smoke from forest fires. The anthropogenic sources of nanoparticles include, in addition to the intentional production, unintentional formation through combustion, tire wear, in the subway and during welding. Due to the rapid development of nanotechnology and the unintentional formation during processes in the industrial society, the potential for human exposure to nanoparticles has increased. As seen in **Figure 7**, nanoparticles are in the same size-range as cellular structures, which can potentially lead to alterations in the interactions with cells and cellular molecules. As adverse health effects of nanoparticles have been observed, concerns regarding the safety of nanoparticles have been raised[90,91].

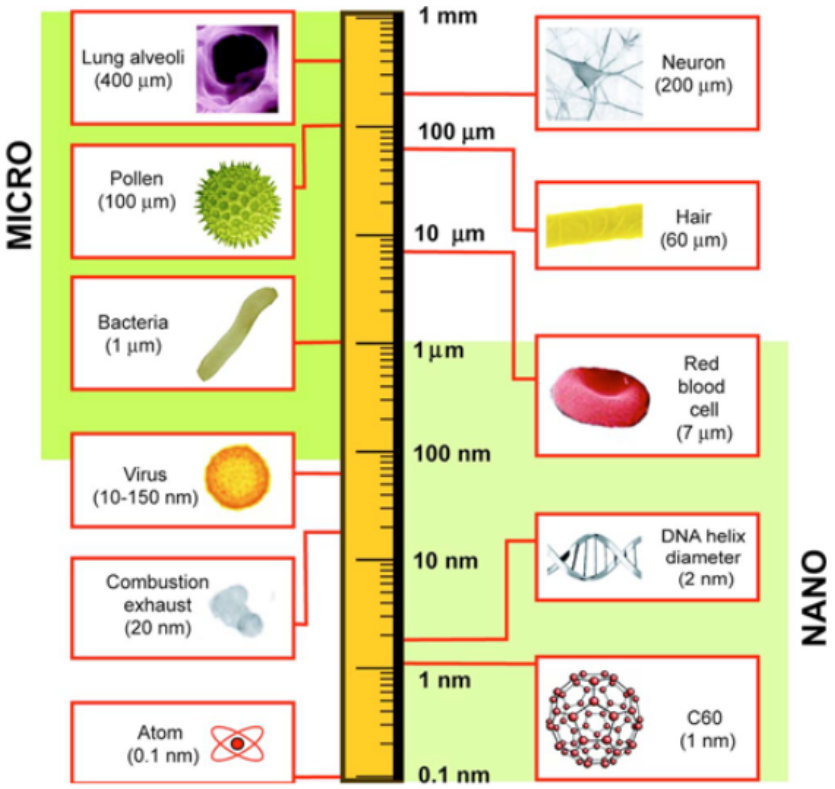

Figure 7. Nano- and microsized materials in a logarithmic length scale for a comparison with the size of biological molecules and cells, reprinted from Buzuea *et al.*[89] with the permission from AIP Publishing LLC. Copyright 2007, American Vacuum Society.

4.2 DEFINITIONS OF NANOMATERIALS

There are no harmonised international definitions of nanomaterials or nanoparticles. Generally, a nanomaterial is defined as a material with at least one dimension in the size-range between 1–100 nm, while a nanoparticle is often defined as an object with all dimensions between 1–100 nm. These general definitions are used within this thesis. According to the European Commission, a more specific definition of a nanomaterial is:

"'Nanomaterial' means a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm–100 nm."[92]

By this definition, particle, agglomerate and aggregate are further defined as:

"(a) 'particle' means a minute piece of matter with defined physical boundaries;
(b) 'agglomerate' means a collection of weakly bound particles or aggregates where the resulting external surface area is similar to the sum of the surface areas of the individual components;
(c) 'aggregate' means a particle comprising of strongly bound or fused particles."[92]

The definition above includes both naturally occurring and anthropogenic nanomaterials. The term *engineered nanomaterial* is often used to describe an intentionally produced nanomaterial with specific properties.

4.3 ENGINEERED NANOMATERIALS AND THEIR APPLICATIONS

Nanotechnology enables engineering at the nanoscale to produce materials with attractive properties. Nanomaterials include materials with different chemical compositions and shapes, such as nanofibers, nanorods, nanospheres, nanotubes and nanosheets. Commonly used materials include carbon, silica, different metals and metal oxides, ceramics and polymers. Due to the diversity in both shape and composition within nanomaterials, and the possibility to tune these properties for the desired purpose, the application areas are of wide variety.

The global market for nanotechnology products was approximately 254 billion US dollars in 2009 and it is estimated to reach 3000 billion US dollars in 2020[93]. In an inventory of nanotechnology-based consumer products presented by The Project on Emerging Nanotechnologies, were 1628 nanoproducts listed as of October 2013[94]. The most common product category was found to be products within health and fitness, and the major materials reported were silver (Ag), titanium and carbon. Silver nanoparticles can be used for antibacterial and anti-odour functions in sports clothing or for wound dressing in biomedical applications[94,95]. Titanium dioxide (TiO_2) nanoparticles have versatile properties and can be found in cosmetic products, sun screens, food and paints[96]. Nano-sized carbon black is one of the most produced nanomaterials and it is used in the production of rubber products and also as a pigment. Furthermore, zinc oxide (ZnO) nanoparticles are highly attractive for various applications, partly due to their optical properties. Copper (Cu) and copper oxide (CuO) nanoparticles are used for antimicrobial purposes in surface coatings, woods, textiles and paints[97]. Furthermore, nanotechnology can be applied within the environmental technology area for water purification, to decrease pollution and capture carbon dioxide[98,99].

4.3.1 Nanomedicine

Nanomedicine is a promising and fast developing field that covers several biomedical applications using engineered nanomaterials. Nanoparticles can be designed for efficient drug and gene delivery, diagnosis, *in vivo* imaging, or combinations of these functions. Moreover, applications of nanomaterials include biomedical implants, biosensors and tissue regeneration.

Liposomes, polymers and metal nanoparticles are commonly applied entities in nanomedicine and several nanomedicines are evaluated in clinical trials, whereas some have already reached the market[100]. Combined therapeutic and diagnostic properties, so called theranostics, is emerging as a promising approach to develop personalised medicine with the possibility to monitor drug distribution and treatment progress[101]. This is appealing not at least in cancer treatment. Nanosized drug-delivery systems can be used to improve the solubility and the pharmacokinetics of

conventional drugs, with the aim to increase the therapeutic index. They offer possibilities to improve and control the biodistribution of the drug, by passive and active targeting with ligands, as well as control of drug release upon internal or external stimuli. The passive targeting of nanoparticles to tumour tissue occurs via the enhanced permeability and retention (EPR) effect, which is caused by the enlarged fenestrations between endothelial cells, as well as impaired lymphatic drainage of tumour tissue, leading to a longer retention of nanoparticles[102].

Gene therapy is a therapeutic approach based on the delivery of nucleic acids, either DNA or RNA, to regulate gene function. Successful gene therapy is dependent on efficient and non-toxic delivery vectors, as naked/unmodified DNA or RNA will be degraded by nucleases or rapidly excreted. Owing to the high loading capacity and the potential ability to control their biodistribution, nanoparticles are of interest as vectors for the delivery of genes. Gold nanoparticles[103], carbon nanotubes[104], dendrimers[105] and silica nanoparticles[106] are examples of nanomaterials that have been explored for application as gene delivery systems. Even though gene therapy is a promising therapeutic approach for several diseases, inefficient gene delivery and toxic vectors are still challenges to overcome. Barriers that need to be considered are; the stability of the DNA/RNA vector complex, undesired enzymatic degradation, rapid renal clearance, unspecific toxicity, cellular internalisation, endosomal entrapment and lysosomal degradation, as well as the nuclear import in the case of DNA delivery[107].

4.3.2 Silica nanoparticles

Mesoporous (materials with pore diameters between 2 and 50 nm) silica (silicon dioxide) nanoparticles have gained a lot of attention for usage within nanomedicine, as they are presenting several interesting properties including tuneable diameter and pore size, high surface area and good biocompatibility[108]. Silica has large amounts of silanol (Si-OH) groups on the surface, which by chemical modifications can be functionalised with different organic groups with desired function. For example, targeting ligands including peptides, antibodies or small molecules such as folic acid can be conjugated to increase the active targeting to tumour tissues. Moreover, surface modification with polyethylene glycol (PEG) chains can be used to reduce non-specific binding of proteins, and thereby avoid clearance by the reticuloendothelial system, resulting in a longer blood circulation time[109]. The structure of the mesoporous silica nanoparticle allows for loading of molecules/drugs within the pores, and by applying so called gatekeepers, the release of the cargo can be controlled. For instance, pH or redox state sensing entities can be applied to control the release upon stimuli[110,111].

4.4 NANOTOXICOLOGY

Along with the increased spread of nanomaterials in the society, questions regarding the safety of these materials have been raised[90,91]. The properties of nanoparticles that make them attractive for applications can also alter the interactions with biological systems and bring on adverse health effects. Their small size can lead to alterations in exposure, translocation, cellular uptake and intracellular fate. Nanoparticles may have the potential to travel inside organisms and penetrate tissues and cells in a greater extent compared to larger particles and bulk form. The term *nanotoxicology* has been proposed to describe the branch of toxicology that comprises research on toxic effects of nanomaterials[112]. One important aspect of nanotoxicology is that materials in the nanoscale may be more toxic compared to their equivalent bulk form. However, the nanosize *per se* do not necessarily imply a hazard, and not every nanomaterial is toxic. It has been argued that there are no nano-specific mechanisms that drive the toxicity of certain nanoparticles[113]. However, there are certain matters that need to be considered in nanotoxicology. Generally, in toxicology the dose makes the poison. Dose metric is of consideration in nanotoxicology and it has been suggested that particle surface area is an appropriate dose metric to be used in nanotoxicology studies[12]. In addition to dose, particle characteristics including the size, shape and surface properties are important factors influencing the toxicity. Much knowledge can be obtained from previous studies on particle toxicology, particle kinetics, epidemiological studies on airborne particles and also from the field of metal toxicity in the case of metal nanomaterials.

Understanding the interactions of nanomaterials with biological systems is crucial for risk assessments and a safe development of nanotechnology products, not least for safe and effective nanomedicine. This includes understanding of the exposure routes, possible translocation to other organs, cellular uptake and mechanisms of toxicity.

4.4.1 Exposure to nanoparticles

The public can be exposed to nanoparticles that are either formed naturally, during combustion or during other unintentional formation processes. In addition, exposure to nanoparticles can occur via usage of consumer products containing nanoparticles or via the release of nanoparticles from these products. Furthermore, occupational exposure to nanoparticles is an important health risk for workers within mining, welding or other activities where nanoparticles are formed intentionally or unintentionally. Routes of exposure depend on the origin, production or application of the nanoparticle or nanoparticle product. Exposure can occur via inhalation, ingestion and dermal exposure both for the general public and in occupational settings, while injection and implants are other possible exposure routes when applying biomedical products.

Lung exposure
Exposure to ambient particles is known to increase the risk for cardiovascular disease and increase mortality and morbidity[114]. Nanosized particles are considered to contribute more to the negative health effects compared to the larger particles.

Upon inhalation, particles deposit in the lung depending on their size, composition and breathing conditions such as breathing strength. The mechanisms that determine the site of deposition include sedimentation (gravitational forces), inertial impaction, interception (contact with the airway surface), electrostatic forces and diffusion. For nanoparticles, diffusion is the most important mechanism[12]. In a mathematical model developed by the International Commission on Radiological Protection, are inhaled particles, depending on their size, predicted to deposit in different mass proportions in the different regions of the respiratory system[12,115]. According to this model, nanoparticles can deposit in all three regions (the upper respiratory tract, the tracheobronchial region and the alveolar region) of the human respiratory system, as shown in **Figure 8**. Single particles with a size around 1 nm, and larger particles around 10 μm, deposit predominantly in the upper part of the respiratory system. Particles with a size of 20 nm deposit mostly in the alveolar region (approx. 50%) as well as in the upper region and the tracheobronchial region (approx. 15% in each of them). Particles with a size of 5 nm deposit in equal proportions (approx. 30%) in each of the three regions.

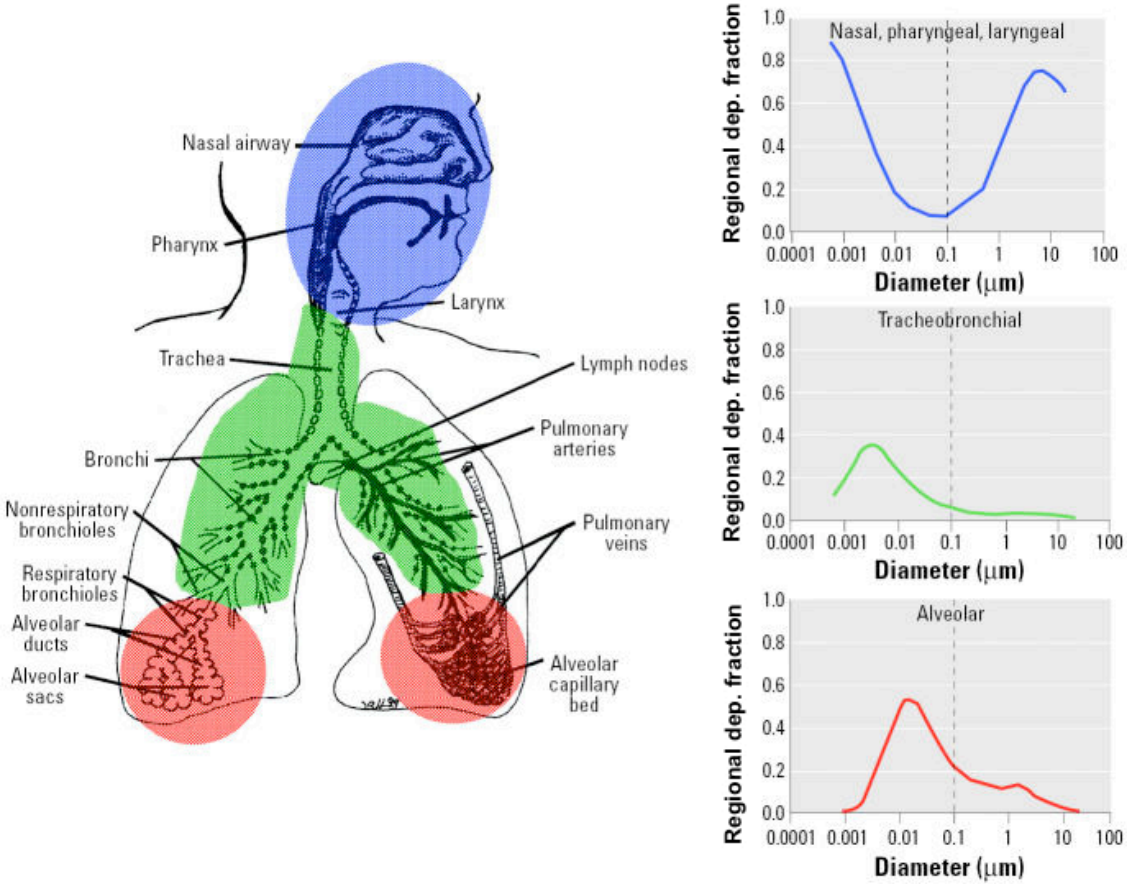

Figure 8. Illustration of size-dependent deposition of particles in the lung, predicted by a model developed by the International Commission on Radiological Protection 1994[115]. The figure is adapted from Oberdörster *et al.*[12], with the permission from Environmental Health Perspectives.

The clearance of the inhaled particles depends on the site of the deposition and also the particle characteristics. Certain particles can be cleared via chemical processes and dissolution, which are processes that can be rather independent on the region of deposition. Mucociliar clearance of deposited particles occur in the conductive airway where the particles become trapped in the mucus and are transported via ciliary movement up to the pharynx to be swallowed or coughed out[116]. The main clearance mechanism in the alveolar region is via phagocytosis by alveolar macrophages[116]. Upon phagocytosis of the particles, macrophages move to the ciliated regions and are cleared via mucociliar clearance. The retention time is longer for particles that deposit in the lower region of the respiratory tract[117]. While the phagocytic processes occur in a couple of hours, the gradual movement towards mucociliar clearance can take up to 700 days in humans[12]. It has also been shown that the phagocytic efficiency is size-dependent with small nanoparticles having the potential to escape the macrophages[118]. The length of nanofibers can also affect the phagocytic clearance. An increase of length of the nanofiber can impair the clearance, following the paradigm of fibre toxicity with similarities to asbestos[119]. Failure in phagocytic clearance and a longer retention time could potentially lead to increased inflammation, cellular interactions and uptake by epithelial cells and further translocation of the nanoparticle to the circulatory system and other organs. The extent of translocation is however debated and conflicting findings are presented in the literature[117,120-122]. Wiebert *et al.*[123] showed that less than 1% of inhaled carbon nanoparticles were translocated from the lung in healthy human subjects.

Oral exposure

Nanoparticles in food, toothpaste and cosmetics, as well as inhaled and mucociliary-cleared nanoparticles, can reach the gastro-intestinal tract. Thus, the complex gastro-intestinal tract, acting as both a site for exchange of macromolecules and a barrier, is a potential entry route for nanoparticles. Translocation across the gastro-intestinal mucosa can occur via endocytosis by epithelial cells, transcytosis by M-cells in the Peyer's patches, via persorption through gaps in the epithelium or potentially by paracellular uptake[124]. The rate of persorption through the mucous layer has been shown to be dependent on the size, with smaller nanoparticles crossing the layer faster[125]. After reaching the sub-mucosal tissue, nanoparticles can enter the lymphatic system and be further translocated to the blood and other organs[126].

Dermal exposure

Dermal exposure to nanoparticles can occur unintentionally via environmental or occupational exposure, or intentionally by usage of consumer products such as sunscreens and cosmetics containing nanoparticles. The skin consists of several layers that form a barrier to prevent the entry of toxic substances and pathogens into the body. Generally, the skin is considered to be a good barrier preventing nanoparticle entrance with several studies showing that nanoparticles do not penetrate intact skin[89,127,128]. However, a study by Murugan *et al.*[129] showed that TiO_2 nanoparticles can lodge in the hair follicles, but further penetration was not shown. The potential penetration of nanoparticles through damaged or flexed skin is unclear[129].

4.4.2 Cellular uptake and mechanisms of toxicity

Interactions of nanomaterials with living cells may cause toxic effects. Undesired effects include cytotoxicity, morphological and structural changes and genotoxicity. Although the exact toxic mechanisms are not fully understood, key events in the toxicity of nanomaterials are considered to be oxidative stress, DNA damage and inflammation[130].

Cellular uptake

The passage of molecules and particles through the cell membrane can occur via a passive or active transport. Most particles and larger macromolecules enter the cells by endocytosis. Phagocytic cells are able to take up larger particles via phagocytosis, a specific type of endocytosis, whereas non-phagocytic cells utilise different pathways of pinocytosis to mediate cellular uptake. Upon cellular uptake, nanoparticles can end up in lysosomes, be transported out of the cell, or reach organelles, such as the mitochondria or nuclei. The cellular uptake of particles depends on the physicochemical properties including the particle size, shape, surface properties and modifications such as possible target ligands, as well as the extent of particle aggregation[131]. In **Figure 9,** potential mechanisms of cellular uptake of nanomaterials are shown, illustrated by Zhao *et al.*[132].

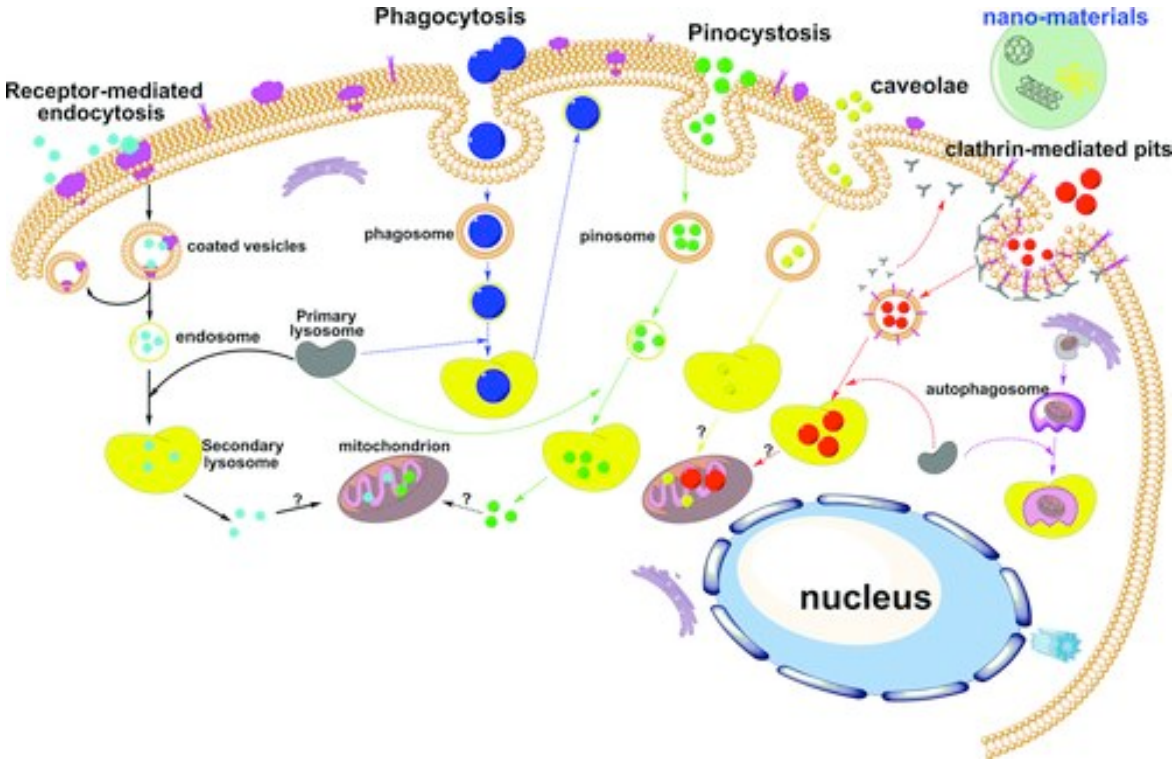

Figure 9. Illustration of potential cellular uptake and intracellular fate of nanomaterials. Reprinted from Zhao *et al.*[132] with permission from John Wiley and Sons.

Oxidative stress

Reactive oxygen species can be generated directly on the nanoparticle surface, as a consequence of particle dissolution, due to direct damage of cellular compartments (e.g. the mitochondria), or as secondary formation due to activation of the immune system or impaired protein function. As described in section 2, cells can make adaptions to increased levels of oxidative stress. The three-tier model of cellular responses to oxidative stress induced by ambient particulate pollutants, as seen in **Figure 10**, can in similarity, be applied as a oxidative stress paradigm for the toxicity of nanoparticles[133]. According to this model, at low levels of oxidative stress (Tier 1), the nuclear factor erythroid 2-related factor 2 (Nrf-2) can induce the expression of antioxidative and detoxifying phase II enzymes such as haem oxygenase 1 (HO-1), glutathione S-transferase (GST) and catalase, to maintain the redox balance. Failure in the antioxidative response, and/or increased ROS levels, may result in inflammatory effects (Tier 2). The inflammatory response is regulated by redox sensitive mitogen-activated protein kinase (MAPK) and NF-κB, which can increase the production of cytokines and chemokines. Further increase of oxidative stress may lead to cytotoxic effects (Tier 3) with the release of pro-apoptotic factors and cell death[133].

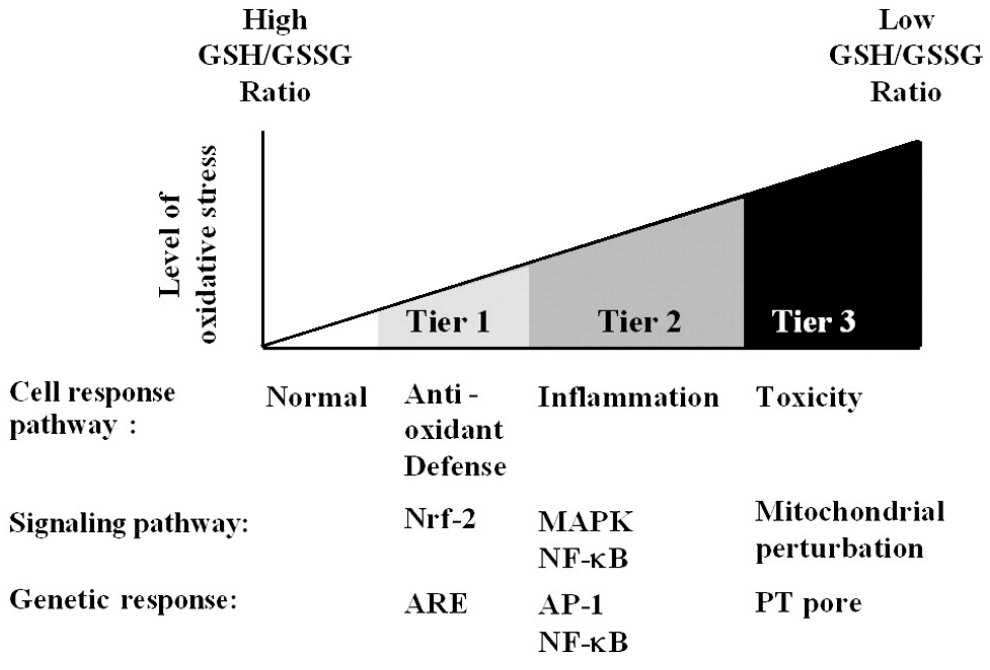

Figure 10. The toxicity of nanoparticles can in similarity to the toxicity of ambient particulate pollutants, be described by an oxidative stress paradigm with three tiers of cellular response depending on the level of oxidative stress. Illustration from Li *et al.*[133], reproduced with permission from Elsevier.

Nanoparticles composed of metal and metal oxides can exert toxicity by extra- or intracellular dissolution resulting in a release of potentially toxic ions. The cell membrane normally acts as a barrier preventing the entrance of ions by regulating the uptake through metal sensing and transport proteins[134]. However, a Trojan horse-type of mechanism is commonly used to describe an endocytic uptake of nanoparticles whereupon reaching the inside of the cell, in the acidic pH (pH in the lysosomes ≈ 4.5), they are dissolved and release toxic ions[135,136]. The release of

transition metal ions, such as Cu, Fe, Cr, Mn, can initiate redox reactions and generation of hydroxyl radicals through Fenton reactions. Other toxic mechanisms of metal ions include non-specific binding to protein ligands and competition with other essential metals resulting in dysfunctional proteins.

In contrast to pro-oxidative effects of nanoparticles, cerium oxide (CeO_2) nanoparticles have been shown to have antioxidative and cytoprotective effects. It was shown in a vein endothelial cell line that nanoceria exerted protective effects on H_2O_2-induced mitochondrial damage and thereby prevented oxidative damage[137]. Korsvik *et al.*[138] demonstrated that the antioxidative effect of CeO_2 mimics the catalytic activity of SOD, with the nanoparticles exhibiting a higher catalytic rate constant, probably due to the large particle surface area with several catalytic sites.

Genotoxicity
Nanoparticle exposure can induce DNA damage either by indirect or direct interactions with the DNA. Indirect DNA damage can be induced when nanoparticles affect proteins important for regulation of the genes or the cell cycle, or by inducing ROS formation and increased oxidative stress levels[139]. Direct interactions with the DNA can occur upon nuclear entrance via the nuclear pores, however, this is most likely limited owing to the structure of the nuclear pore complex. Nonetheless, *in vitro* studies have showed that small nanoparticles (<10 nm) can access the nuclei, e.g. gold nanoparticles (2 and 6 nm)[140] and SiC nanocrystals (4 nm)[141]. Larger nanoparticles have also been detected in the nucleus. For instance, silica nanoparticles with a diameter of 70 nm were shown to enter the nucleus in HaCaT cells (human keratinocytes)[142] while silicon carbide nanoparticles (10–60 nm) were detected in the nucleus in A549 cells (human lung cells)[139]. Larger nanoparticles may have direct interactions with the DNA during the mitotic phase, during which the nuclear membrane breaks down.

Indirect DNA damage can occur due to increased levels of ROS, as described in section 2.1.1. Since both nanoparticles and ROS can potentially inactivate proteins, indirect DNA damage can also emerge if proteins involved in DNA repair, replication or transcription are damaged[139]. Moreover, some nanoparticles including silica, have been shown to induce epigenetic changes[143].

Inflammation
Activation of the inflammatory system is a normal cellular response to invading pathogens, particles, cell injuries, chemicals or other potential harmful substances. The innate immune system provides a rapid inflammatory response, potentially within minutes upon triggering. Activation of phagocytic cells upon nanoparticle engulfment can initiate cascades of pro-inflammatory molecules (cytokines and chemokines) to recruit and activate other immune cells. In addition, release of ROS through activation of NADPH-oxidase and oxidative burst can occur.

Nanoparticles can be designed to target or to avoid the activation of the immune system, depending on the application and the desired effect. Non-functionalised carbon nanotubes were shown not to trigger engulfment or oxidative burst in macrophages, whereas surface modifications with phosphatidylserine (PS) increased

the uptake by macrophages[144,145]. Certain nanoparticles can also act in an immunosuppressive way and decrease the ability of the body to recognise pathogens[146]. For instance, Lundborg *et al.*[147] showed that ingestion of ultrafine carbonaceous particles by macrophages impaired their ability to phagocyte. Such impairment could lead to increased susceptibility to infections.

4.4.3 Physicochemical properties influence the biological interactions

The biological responses upon exposure to nanoparticles can be different for each type of nanoparticle as they depend on the physicochemical properties of the nanoparticle. Particle characteristics influencing the cellular interactions and toxicity include size, chemical composition, shape, surface area, surface charge, crystallinity, solubility, agglomeration state and possible surface coating. The surface properties of the particle will affect the binding of proteins and other biomolecules when in contact with biological fluids. This protein corona changes the hydrodynamic size and the surface charge, and it will dictate the biological fate and the toxicity of the particle[148]. Therefore, thorough investigation of physicochemical properties is essential when evaluating toxicity and potential biomedical use of nanomaterials.

5 RESEARCH AIMS

The general aim of this thesis was to study oxidative stress, DNA damage and toxic mechanisms induced by either kidney disease or nanoparticles. Studies on CKD and dietary supplementation *in vivo*, as well as nanoparticle exposure *in vitro* are included.

The specific aims of the studies included in this thesis are as follows:

– Assess the levels of oxidative stress and DNA damage in minor salivary glands, as well as investigate the correlation with saliva production, inflammation and uremic markers in patients with CKD. (Study I)

– Investigate the effects of a dietary supplement containing sea buckthorn extract on oxidative stress and DNA damage in minor salivary glands, as well as saliva production, inflammation and uremic markers in patients with CKD. (Study II)

– Compare the cytotoxicity of a range of nano- and microsized particles and screen for particularly toxic particles *in vitro*.

– Investigate differences in the cytotoxicity of selected nanoparticles among different cell types.

– Investigate the toxicity of Cu-based nanoparticles (Cu and CuO nanoparticles) and study toxic mechanisms including oxidative stress, DNA damage, mitochondrial damage and cell death *in vitro.* (Study III)

– Study the biocompatibility of amine-modified silica nanoparticles and examine their potential use as vectors for delivery of plasmid DNA *in vitro*. (Study IV)

6 STUDY APPROACH

The first part of this thesis is based on an observational study investigating the levels of oxidative stress, DNA damage and inflammation in CKD patients, and an intervention study of a dietary supplement containing a sea buckthorn extract in the same patient group. The second part of this thesis is based on *in vitro* studies on toxicity, biocompatibility and application of nanoparticles.

6.1 STUDY I AND II: DNA DAMAGE AND OXIDATIVE STRESS IN CHRONIC KIDNEY DISEASE PATIENTS

The main aim of Study I, the observational study, was to assess the levels of DNA damage and oxidative DNA damage (as a biomarker for oxidative stress) in salivary glands, as well as to investigate the correlation with saliva production, inflammation and uremic markers. It has previously been shown that CKD patients have higher levels of DNA damage and oxidative stress in PBMC, and that CKD patients often suffer from impaired oral health. Thus, it was hypothesised that elevated levels of DNA damage and oxidative DNA damage can also be found in the salivary glands of CKD patients, with potential correlation with hyposalivation. No previous studies on DNA damage in the salivary glands in this patient group have been found in the literature. The patient group of 79 CKD patients, of whom 69 were dialysis patients (66 haemodialysis patients and 3 peritoneal dialysis patients) and 10 were predialysis patients (CKD patients not yet on dialysis), was recruited from the Karolinska University Hospital and other dialysis units in Stockholm. Control patients were recruited from the public dental service clinic in Solna and were age- and sex-matched to the CKD patients. Biopsies of the minor salivary glands were collected by incision and frozen prior to DNA damage analysis with the comet assay. Blood and saliva samples were collected as well.

In Study II, the intervention study, the main aim was to study the effects of a dietary supplement containing an oil extract of sea buckthorn on oxidative stress and DNA damage in salivary glands in haemodialysis patients. In addition, effects on saliva production, inflammation and uremic markers were also investigated. Since previous studies on sea buckthorn have shown antioxidative effects *in vitro*[55,56] and beneficial effects on mucous membranes *in vivo*[9], it was hypothesised that supplementation with sea buckthorn extract would have beneficial effects on DNA damage and oxidative DNA damage in the salivary glands as well as improve oral health in terms of increase of saliva production. The study was a randomised, double-blinded crossover study with 2 × 8 weeks treatment periods. The study design is shown in **Figure 11**. A four-week wash-out period was applied in between the treatment periods to avoid carry-over effects. The study subjects were 63 haemodialysis patients recruited from the Karolinska University Hospital in Stockholm. The patients were randomly assigned in two patient groups with different treatment sequences. The first group received sea buckthorn capsules during the first study period and placebo capsules during the second period, while the other group received placebo capsules in the first period and sea buckthorn capsules in the second period. The

intake was instructed to four capsules per day. The content of the sea buckthorn capsules and the daily dose are shown in **Table 2**. The daily doses of vitamin A and vitamin E were both slightly less than half of the recommended daily intake in Sweden, see section 2.2.2. Appointments to collect salivary gland biopsies, blood and saliva samples were conducted before and after each treatment period, i.e. in total four appointments per patient. The comet assay was used to assess the levels of DNA damage and oxidative DNA damage in the salivary glands.

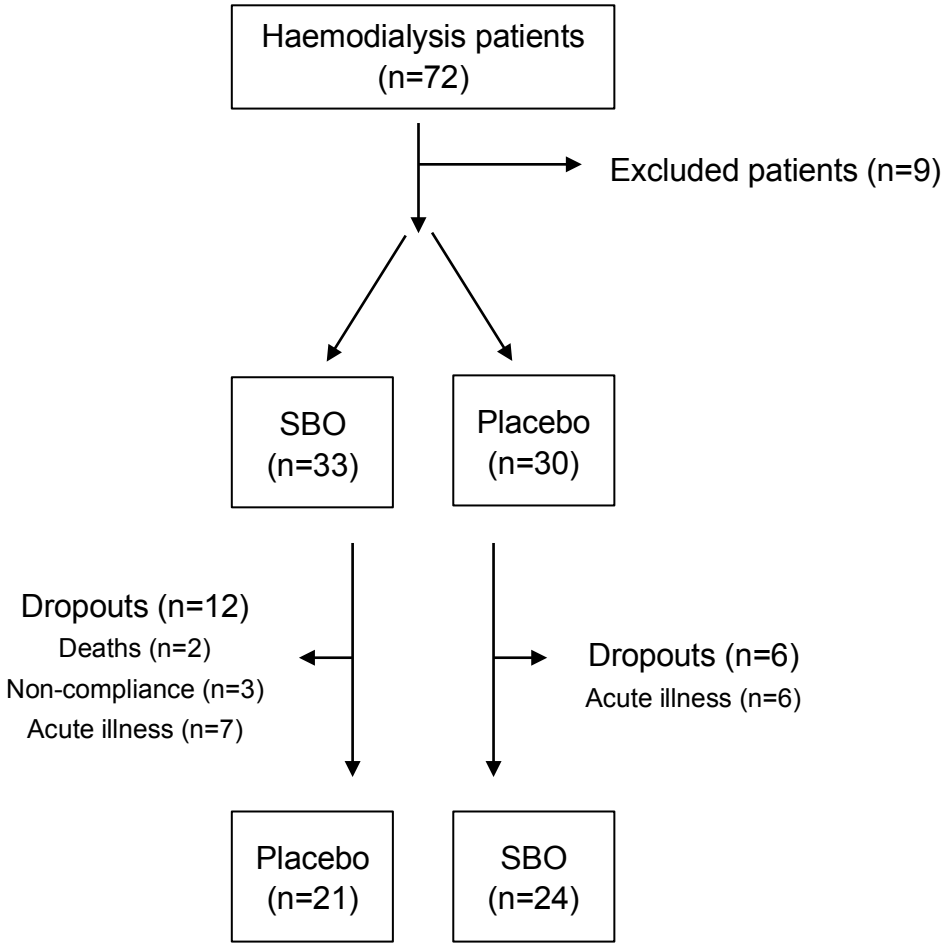

Figure 11. A scheme of the study design in the intervention study (Study II), wherein the effect of supplementation with the sea buckthorn oil (SBO) extract in haemodialysis patients was evaluated.

Table 2. Capsule content and daily dose of the sea buckthorn supplement.

	1 SBO capsule (500 mg)	Daily dose (4 capsules, 2 g)
Oleic acid (C18:1 n-9)	124 mg (24.8%)	496 mg
Palmitoleic acid (C16:1 n-7)	97 mg (19.5%)	388 mg
Linoleic acid (C18:2 n-6)	92 mg (18.4%)	368 mg
α-linolenic acid (C18:3 n-3)	63 mg (12.6%)	252 mg
Vitamin E	931 μg	3.7 mg
Vitamin A	88 μg	352 μg

6.2 STUDY III AND IV: TOXICITY AND BIOCOMPATIBILITY OF NANOPARTICLES

The aims of the second part of this thesis were to identify particularly toxic nanoparticles as well as biocompatible nanoparticles, by screening a range of nano- and microparticles, and to further examine the toxic mechanisms. Initially, a range of 37 different particles was screened for their cytotoxicity in the human lung cell line A549 using the trypan blue exclusion assay. Subsequently, a selection of the particles was further compared in five additional human cell types to explore any cell type-specific cytotoxicity. The studied cell types were A549 (lung carcinoma cell line), HL60 (acute myeloid leukaemia M2 cell line), Jurkat cells (T lymphocytic cell line), K562 (chronic myeloid cell line), PC-3 (prostate adenocarcinoma cell line) and lymphocytes isolated from healthy blood donors.

Based on the high cytotoxicity observed for Cu-based (Cu and CuO) nanoparticles, their toxicity was further explored in Study III. Dose-response relationships as well as toxic mechanisms including DNA damage, oxidative DNA damage, cell death type, mitochondrial damage and ROS formation were investigated after exposing the cultured cells to the nanoparticles. Since previous studies have shown that nanoparticles can translocate via the blood to other organs, the HL60 cell line was chosen as a model to assess toxicity to blood cells. For comparison, the toxic effects of dissolved Cu from the easily soluble $CuCl_2$ were also investigated for comparison as several studies have suggested that the toxicity of CuO nanoparticles is partly mediated via the release of Cu ions. Finally, particle characteristics including size measurements, agglomeration and the release of Cu from the particles were also investigated.

In Study IV, amine-modified silica nanoparticles were investigated for their biocompatibility and potential use as vectors for gene delivery. Nonporous silica nanoparticles were compared to mesoporous silica nanoparticles in their efficiency to deliver plasmid DNA in the human breast carcinoma cell line MCF-7. The biocompatibility of the nanoparticles was studied using the MTT assay and the colony formation efficiency assay. The plasmid DNA delivery efficiency was measured using luciferase-encoding plasmid DNA with subsequent measurement of luciferase activity. In addition, as the protein corona has been shown to play an important role in both toxicity and delivery efficiency of nanoparticles, it was investigated how the presence or absence of serum in the cell medium influences these features. Particle uptake was studied using transmission electron microscopy (TEM).

7 METHODS

This section presents a brief summary of the methods used for assessing the DNA damage in the salivary glands, as well as for assessing the toxicity and biocompatibility of the nanoparticles, including the particle characterisation methods performed by collaborators at the Royal Institute of Technology and the electron microscopy unit at Karolinska University Hospital. More detailed information and statistical methods can be found in each publication in the appendix.

7.1 COMET ASSAY

The comet assay (also known as single-cell gel electrophoresis) is a method to measure DNA damage in cells. Different cell types from blood, tissue or cell cultures can be analysed. The alkaline version, first described by Singh *et al.*[149], is the most commonly used version to detect strand breaks and alkali-labile sites in the DNA. The method can be modified with additional steps of enzyme treatment to detect specific DNA lesions. Here, the alkaline version of the comet assay was used to detect strand breaks and alkali-labile sites (both together are in the thesis referred to as DNA damage). The method was modified with an additional step using FPG to enable detection of DNA that has been damaged by oxidation (in this thesis referred to as oxidative DNA damage). FPG is a bacterial repair glycosylase which detects oxidised purines, mainly 8-oxo-G, but also fapy-G and fapy-A. The enzyme exerts its glycosylase activity by cleaving the glycosidic bond between the base and the sugar when encountering an oxidised base. This site is then cleaved to an apurinic site due to the AP-lyase activity of the bifunctional FPG[150]. Other lesion-specific enzymes can also be used in the comet assay; examples are hOGG1, EndoIII and UNG.

A scheme of the basic steps of the comet assay is shown in **Figure 12.** The first step is to retrieve a single cell suspension. In Study I and II, in which salivary gland tissue was analysed, this was achieved by tissue homogenisation using a dounce pestle. In Study III cultured cells were used and cell suspensions were obtained after centrifugation and resuspension of exposed cells in phosphate-buffered saline (PBS). The cell suspensions are then mixed with agarose, spread on microscopic slides and the gels are left to solidify on ice. The cells are then put in lysis, during which the membranes and cell constituents are broken down, and the structure that remains is here referred to as the nucleoid. If specific DNA lesions are to be analysed, the slides are then incubated with the specific enzyme, in this thesis FPG, to allow detection and creation of apurinic sites at the damaged sites. The apurinic sites are then cut to strand breaks, by either lyase activity or during the following alkaline treatment. During the alkaline treatment (pH >13), the DNA is unwound and alkali-labile sites are hydrolysed to strand breaks. The alkaline treatment is followed by electrophoresis during which the strand breaks allow the supercoiled DNA loops to relax and migrate from the nucleoid towards the anode. This migration, dependent on the level of DNA damage, forms structures resembling comets; with the heads containing supercoiled DNA and the tails containing migrated DNA loops.

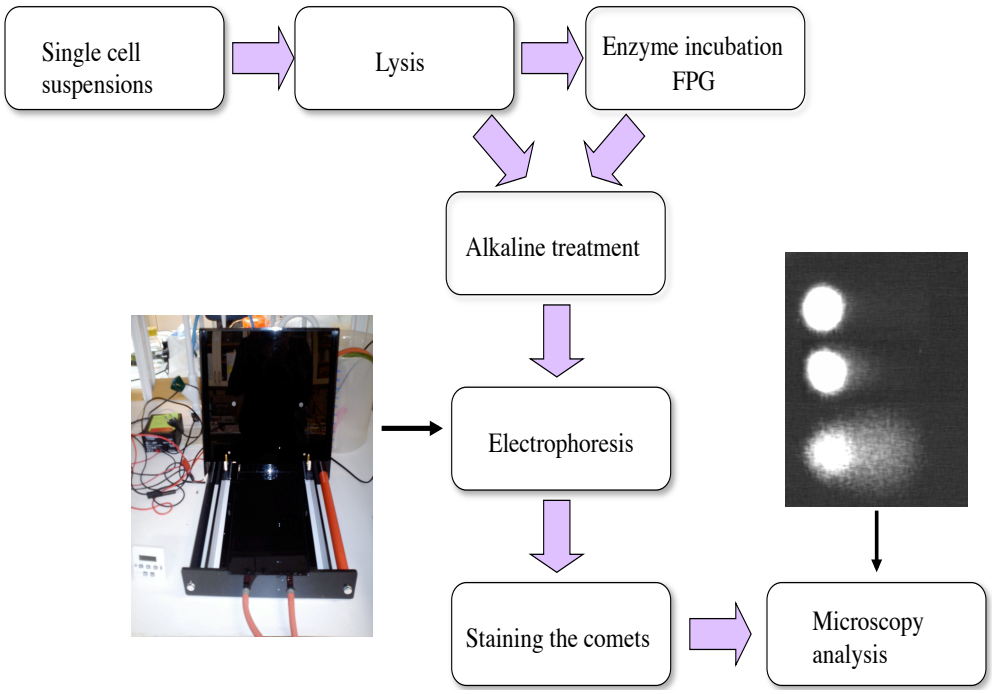

Figure 12. Scheme of the basic steps performed in the comet assay to analyse DNA damage in single cells.

To enable visualisation of the comets and quantification of the DNA damage, the slides are stained after neutralisation and fixation. Staining in this thesis was carried out with ethidium bromide, a fluorescent dye that binds to DNA. Other frequently used dyes are SYBR®safe, SYBR®gold and DAPI (4',6-diamidino-2-phenylindole). After the staining, the comets can be visualised using a UV-fluorescent microscope and the percentage of the amount of DNA in the tail compared to the head can be analysed. Other units that can be used to estimate the level of DNA damage are tail moment and tail length.

The level of oxidative DNA damage (upon using FPG) is calculated by subtracting the value of the non-FPG treated cells, from the value of the FPG-treated cells.

7.2 ANALYSIS OF CYTOTOXICITY AND TOXIC MECHANISMS

A variety of cell viability assays can be used for toxicity testing as well as for counting cell numbers for culturing or performing other cell-based assays. The following sections briefly present the assays used in this thesis to study cytotoxicity and toxic mechanisms of nanoparticles.

7.2.1 Trypan blue exclusion assay

The trypan blue exclusion assay is a commonly used method to measure cell viability. Cells with intact cell membranes are able to exclude the trypan blue dye, while damaged cells (non-viable) are not and are therefore stained blue. From counting the cells using a microscope, the percentage of dead cells can be calculated

as a measure of cytotoxicity. The method was used in this thesis to study the cytotoxicity of nanoparticles in Study III.

7.2.2 MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is also a commonly used method to assess cell viability. MTT is a colometric assay that measures the metabolic activity of cells. The yellow tetrazolium salt is reduced by dehydrogenases in the mitochondria to the insoluble and purple formazan. Absorbance measurements, using a spectrophotometer, enables assessment of cell viability by relating the metabolic activity of cells in exposed populations to unexposed populations. The MTT assay was used in Study IV to measure the cell viability in MCF-7 cells after exposure to amine-modified silica nanoparticles complexed with plasmid DNA.

7.2.3 Resazurin assay

The resazurin assay (Alamar blue) is another method to evaluate cell viability. The dye is converted to fluorescent resorufin upon reduction in metabolically active cells, which enables the assessment of cell viability by fluorescence or absorbance measurements (less sensitive). This method was applied to assess the cytotoxicity of nanoparticles. However, the results showed that some of the nanoparticles interfered with the dye (e.g. the ZnO nanoparticles), hence other viability assays were used instead.

7.2.4 Annexin V-PI assay

The above described cell viability assays do not reveal the type of cell death that has been induced. The Annexin V-PI assay is a method which enables distinction between necrotic and apoptotic cells. Annexin V is a protein with high affinity for PS, a membrane phospholipid which is located in the inner leaflet of the cell membrane in living cells. Cells undergoing apoptosis expose PS at an early stage of the event to the extracellular environment to mark the cell for phagocytosis. By using Annexin V conjugated with the fluorochrome FITC, the translocation of PS can be detected by flow cytometry. Propidium iodide (PI) is a fluorescent DNA-binding molecule, which can be excluded by viable cells in contrast to cells with damaged cell membrane. That is, when using the Annexin V-PI assay to analyse cell death type, viable cells are not stained with any of the dyes, whereas early apoptotic cells are stained with FITC-Annexin V, and necrotic and end-stage apoptotic cells (with lost cell membrane integrity) are stained with both FITC-Annexin V and PI. In early necrotic cells are the cells first only stained with PI as the PI molecule is smaller and penetrate the cells more easily compared to Annexin V. The Annexin V-PI assay was used in Study III to analyse the type of cell death induced by Cu-based nanoparticles.

7.2.5 Colony formation efficiency assay

The colony formation efficiency assay is a method to assess cytotoxicity by measuring the ability of single cells to form colonies after exposure to any toxic agent. These cells are called clonogenic cells. This assay was used in Study IV to assess the effect of amine-modified silica nanoparticle on proliferation in MCF-7 cells.

7.2.6 Mitochondrial damage

Damaged mitochondria with loss in mitochondrial membrane potential can lead to energy depletion, disturbances in the electron transport chain, increased levels of oxidative stress and cell death. The fluorescent probe tetramethyl rhodamine methyl ester (TMRM) can be used to assess mitochondrial damage. This lipophilic and cationic dye accumulates in the mitochondria depending on the mitochondrial membrane potential. A loss in mitochondrial membrane potential leads to less accumulation of the dye and decreased fluorescence. Incubation with TMRM and subsequent FACS analysis was performed in Study III to evaluate the ability of Cu-based nanoparticles to induce loss of mitochondrial membrane potential.

7.2.7 ROS formation

One common molecule that can be used to assess ROS *in vitro* is the 2',7'-dichlorfluorescein-diacetate (DCFH-DA) probe. This non-fluorescent molecule can easily enter the cell, and once inside it is deacetylated by esterases to DCFH. Upon reaction with ROS, the fluorescent compound DCF is formed, which can be detected by fluorescence measurements. The oxidation of DCFH can be rapidly performed by OH•, RO_2• or NO_2•, but not by O_2^-• or H_2O_2 alone (peroxidases or transition metal ions can be used as catalysts)[1]. DCFH-DA was used in Study III to assess intracellular ROS formation after exposing cells to Cu-based nanoparticles.

As it has been shown that ROS can be formed directly on the surface of nanoparticles, DCFH-DA was also used in Study III to study ROS formation without cells. In this acellular setting, the cleavage of DCFH-DA to DCFH can be performed by addition of NaOH prior to incubation with the nanoparticles.

7.3 PARTICLE CHARACTERISATION

7.3.1 Transmission electron microscopy

Electron microscopy is an imaging technique that uses an electron beam to visualise an object. The short wavelength of electrons enables a much higher resolution compared to normal light microscopy, which uses photons with longer wavelengths. Electron microscopy is therefore extensively used to visualise and obtain information on size and shape of nanoparticles. TEM was applied in Study III and IV to visualise the Cu-based and silica nanoparticles at dry conditions. TEM was also used in Study IV to visualise cellular uptake of silica nanoparticles in the MCF-7 cells.

7.3.2 Photon cross-correlation spectroscopy

In addition to studying the features of nanoparticles under dry conditions, it is also necessary to study their behaviour in solution. Nanoparticles tend to group together and form agglomerates in solution. Photon cross-correlation spectroscopy (PCCS) is a technique using dynamic light scattering (DLS) to study particle hydrodynamic size distributions. In PCCS, two laser beams shine onto the sample solution, and the Brownian motion of particles in solution, caused by collision with atoms or molecules in the liquid, induce fluctuations in the scattering intensities. By analysing the fluctuations it is possible to calculate the size of the particles and agglomerates over time. DLS was used in Study III and IV to estimate the hydrodynamic sizes of the Cu-based nanoparticles and the silica nanoparticles.

7.3.3 Zeta potential

The zeta potential of particles affects their stability and agglomeration in solution. A charged particle in solution is surrounded by ions of opposite charge, forming a double layer with strongly bound ions in the inner layer, also known as the Stern layer, and more loosely attached ions in the outer layer. This double layer creates electrostatical forces upon particle movement. By applying an electrical field and measurement of particle velocity, the zeta potential of the particle can be calculated. The zeta potential depends on the surface charge of the particles, as well as the ionic strength, pH and the composition of the solvent. In Study IV, the zeta potential of the silica nanoparticles, with and without complexed plasmid DNA, was measured using DLS.

7.3.4 Atomic absorption spectroscopy

Atomic absorption spectroscopy (AAS) is a technique used to quantify a specific element in a sample by volatilising the atoms and subsequently analysing the energy absorption spectrum. In Study III, flame AAS was used to measure the amount of released Cu from the Cu-based nanoparticles into the cell medium.

8 STUDY RESULTS AND DISCUSSION

8.1 STUDY I AND II: DNA DAMAGE AND OXIDATIVE STRESS IN CHRONIC KIDNEY DISEASE PATIENTS

8.1.1 Study I

Elevated levels of systemic inflammation and oxidative stress are well known in CKD patients and implicate a higher risk for cardiovascular disease[4]. In addition, CKD patients often suffer from oral complaints including inflammation in the oral cavity, periodontitis, changes in the saliva production and in the saliva constituent[85,151]. Impaired oral health can contribute to systemic inflammation and an increased risk for cardiovascular disease. Even though high levels of DNA damage and oxidative DNA damage have been previously reported in PBMC from CKD patients[69,152], studies regarding the DNA damage levels in peripheral tissue, such as the salivary glands, are rare. The aim of Study I was to assess the levels of DNA damage and oxidative DNA damage in minor salivary glands, and to investigate correlation with saliva production, inflammation and uremic markers in CKD patients, including both predialysis patients (CKD patients not yet on dialysis) and dialysis patients. Salivary glands were studied as they are essential for maintaining a good oral health. Damage to the salivary glands can cause decreased saliva production, symptoms of dry mouth and increased risk for infections[86]. The levels of DNA damage and oxidative DNA damage were hypothesised to be increased in the CKD patients, and the damage to be correlated with hyposalivation.

The results showed that, with regards to DNA damage analysed with the comet assay, predialysis patients had significantly higher ($p<0.05$) levels of DNA damage (8.0% DNA in tail, median value) compared to matched controls (6.0% DNA in tail), as seen in **Table 3**. These results are in agreement with previous studies showing elevated levels of DNA damage in CKD patients, although in PBMC[69,153]. The DNA damage in predialysis patients was also significantly higher ($p<0.001$) compared to the dialysis patients (5.3% DNA in tail), as seen in **Table 3** and in **Table 4.** In similarity, Corredor *et al*.[154] recently observed higher levels of DNA damage in PBMC of predialysis patients compared to dialysis patients. An explanation to the higher levels of DNA damage in predialysis patients compared to dialysis patients may be that the dialysis treatment removes the toxic compounds that can potentially induce DNA damage. Indeed, the predialysis patients had significantly higher levels of serum urea and leukocyte-particle concentration (LPC) compared to the dialysis patients. However, conflicting results have been presented. Schupp *et al.*[155] reported that there were no significant changes in DNA damage in PBMC upon initiation of haemodialysis, evaluated by both a micronuclei assay and the comet assay. On the contrary to our hypothesis, the dialysis patients showed even lower levels of DNA damage (5.3% DNA in tail) compared to their matched controls (8.3% DNA in tail). These puzzling observations could potentially be explained by a trigger of DNA repair mechanisms induced by the increase pro-inflammatory signals as a result of the interaction of blood cells with the dialyzer membrane. It was previously shown

by Herman *et al.*[156] that spontaneous DNA repair was induced just after dialysis treatment in PBMC.

Table 3. Levels of DNA damage, saliva production, inflammation and uremic markers in predialysis patients and matched controls.

		Predialysis patients				Matched controls				
		Median	Mean	(min–max)	n	Median	Mean	(min–max)	n	Sign.
	Age (years)	57	56	(33–66)	10	57	57	(36–69)	10	0.76
DNA damage										
	DNA strand breaks (%DNA in tail)	8.0	8.9	(5.9–13.8)	10	6.0	6.4	(5.1–9.9)	9	*
	Oxidative DNA lesions (%DNA in tail)	8.0	7.9	(2.4–14.4)	10	8.2	7.8	(6.1–10.1)	9	0.87
Salivary secretion										
	Secretion rate at rest (mL/min)	0.2	0.3	(0.0–0.9)	10	0.3	0.3	(0.1–0.4)	9	0.653
	Secretion rate, stimulated (mL/min)	1.6	1.9	(0.7–4.4)	10	1.8	1.8	(1.1–3.0)	9	0.683
Inflammation parameters										
	IL-6 in saliva (pg/L)	15.5	16.1	(1.3–40.0)	9	5.0	14.5	(1.6–74.4)	8	0.63
	hs-CRP in serum (mg/L)	5.0	10.8	(1.0–67.0)	10	1.0	1.0	(0.0–2.0)	10	**
	Orosomucoid in plasma (g/L)	1.1	1.2	(0.8–2.0)	8	0.7	0.7	(0.6–0.9)	10	***
	Haptoglobin in plasma (g/L)	1.4	1.8	(1.0–3.2)	8	0.8	0.7	(0.3–1.1)	10	**
	LPC in blood (10^9/L)	8.5	9.0	(5.6–14.9)	10	6.1	7.5	(5.7–17.2)	10	0.075
Uremic state										
	Urea in serum (mmol/L)	25.6	26.5	(12.9–36.6)	10	5.8	5.8	(4.9–6.8)	10	***
	Creatinine in serum (μmol/L)	628	626	(341–937)	10	72	73	(54–95)	10	***
	Albumin in serum (g/L)	38	37	(23–48)	10	40	40	(36–43)	10	0.062
	Hemoglobin in blood (g/L)	121	119	(92–135)	10	148	148	(126–165)	10	***

* p<0.05, **p<0.01 and *** p<0.001

Table 4. Levels of DNA damage, saliva production, inflammation and uremic markers in dialysis patients and matched controls.

		Dialysis patients				Matched controls				
		Median	Mean	(min–max)	n	Median	Mean	(min–max)	n	Sign.
	Age (years)	63	62	(25–87)	69	62	63	(35–89)	69	0.821
DNA damage										
	DNA strand breaks (%DNA in tail)	5.3	5.6	(3.4–13.2)	59	8.3	8.8	(4.7–18.4)	66	***
	Oxidative DNA lesions (%DNA in tail)	7.6	8.4	(1.4–24.2)	59	8.2	8.6	(0.9–16.7)	66	0.515
Salivary secretion										
	Secretion rate at rest (mL/min)	0.1	0.1	(0.0–0.5)	68	0.2	0.3	(0.0–1.4)	69	***
	Secretion rate, stimulated (mL/min)	1.1	1.1	(0.0–2.5)	68	1.7	1.8	(0.3–5.2)	69	***
Inflammation parameters										
	IL-6 in saliva (pg/L)	15.0	40.4	(0.2–154.5)	5	5.5	12.9	(0.2–91.5)	53	0.383
	hs–CRP in serum (mg/L)	4.5	8.5	(1.0–77.0)	68	2.0	3.4	(1.0–33.0)	68	***
	Orosomucoid in plasma (g/L)	0.9	1.0	(0.4–2.2)	67	0.8	0.8	(0.4–1.2)	68	***
	Haptoglobin in plasma (g/L)	1.2	1.2	(0.1–2.6)	67	1.1	1.1	(0.2–2.4)	68	0.308
	LPC in blood (10^9/L)	6.9	7.1	(1.8–14.6)	69	6.1	6.6	(4.2–14.0)	69	0.059
Uremic state										
	Urea in serum (mmol/L)	20.5	21.7	(13.3–46.4)	69	5.8	6.0	(3.6–11.2)	67	***
	Creatinine in serum (μmol/L)	716	730	(271–1333)	69	78	79	(48–118)	69	***
	Albumin in serum (g/L)	35	35	(27–44)	68	39	39	(32–46)	69	***
	Hemoglobin in blood (g/L)	125	123	(91–156)	69	146	146	(112–168)	67	***

***p<0.001

With regards to the oxidative DNA damage, no significant differences among the groups were observed. One could speculate that increased activity of DNA repair and antioxidative enzymes are activated upon the enhanced systemic inflammation and oxidative stress in the CKD patients. Indeed, Bibi *et al.*[157] showed that peritoneal dialysis patients had higher levels of salivary peroxidase and SOD, compared to predialysis patients. However, partially in contrast to our findings, antioxidants in saliva and serum were shown to be differently affected by peritoneal dialysis. Uric acid (an important antioxidant in saliva) and the total antioxidant status, were found to be lower in saliva compared to in serum[157]. The dialysis treatment itself can induce ROS formation by activation of neutrophils, which can be caused by a bioincompatible dialyzer or contaminated dialysate. It is possible that, since the circulating PBMC are in contact with the dialysis membrane, they are triggered to release ROS, which can induce activation of the antioxidative defence and DNA

repair mechanisms in the peripheral tissue. Circulating PBMC and peripheral tissue are thus affected differently; while the inflammation and oxidative stress in the blood system is elevated, the level in peripheral tissue might be lower or unaffected due to the upregulated antioxidative defence or DNA repair mechanisms. However, further investigations would be needed to confirm the potentially upregulated repair and antioxidative mechanisms, for example by analysis of DNA repair in the salivary glands or other peripheral tissue. For a less invasive approach, assessment of oxidative stress in the oral cavity can be conducted by analysing 8-oxodG in saliva, or potentially by analysing buccal cells using the comet assay. It would also have been of value to confirm previous findings of higher levels of DNA damage and oxidative DNA damage in PBMC in the present predialysis and dialysis patients.

The salivary secretion rates (both at rest and after stimulation) were, as expected, lower in the dialysis patients compared to the matched controls as well as the predialysis patients. The salivary secretion rate at rest correlated well to the rate after stimulation in both CKD patients and the controls. Previous studies have shown similar results, e.g. Gavalda *et al.*[84] showed a significant decrease in stimulated saliva production among dialysis patients compared to the controls. Kho *et al.*[158] also showed decreased levels of saliva production, both at rest and stimulated parotid saliva. Damage to the salivary gland and/or restrictions in fluid intake were proposed as underlying factors. Hyposalivation could be an explanation to the higher prevalence of oral complaints among CKD patients since the saliva is important for lubrication, bacterial defence, buffering capacity, taste and digestion. No significant correlation between the salivary secretion rates and DNA damage or oxidative DNA damage in the salivary glands was observed.

While all inflammation parameters showed increased levels in the CKD patients compared to the matched controls, all observed differences were not statistically significant. For example, IL-6 in saliva was only measured in a limited number of CKD patients, hence, the difference to the control patients was not statistically significant. The uremic parameters, urea and creatinine in serum, were significantly higher in both predialysis and dialysis patients compared to the matched controls. In contrast, the levels of albumin and haemoglobin were significantly lower in dialysis patients compared to the matched controls. Likewise, the levels in predialysis patients were also lower compared to the matched controls, although only the difference in haemoglobin reached statistical significance. Moreover, there was a significant correlation between haptoglobin and oxidative DNA damage in dialysis patients.

It was additionally found that oxidative DNA damage was sex-dependent; women had significantly higher levels compared to men in the CKD patient group. That is, within the dialysis patients; 9.6% compared to 7.8% DNA in tail and within the predialysis patients; 11.9% compared to 7.6% DNA in tail. Age, smoking habits, and diabetes were not significantly related to the levels of DNA damage or oxidative DNA damage in any of the tested statistical models.

In conclusion, predialysis CKD patients had significantly higher levels of DNA damage in minor salivary glands compared to age- and sex-matched controls. On the contrary, dialysis patients had significantly lower levels of DNA damage in the minor

salivary glands compared to both controls and predialysis patients. No difference in oxidative DNA damage was observed. The results suggest that the DNA in salivary glands is affected differently by the CKD and the dialysis treatment, compared to circulating PBMC. The saliva production, both at rest and stimulated, was lower in CKD patients compared to controls. Inflammation and uremic markers were elevated compared to controls. There was a correlation between haptoglobin and oxidative DNA damage in dialysis patients. No significant correlation between the DNA damage or oxidative DNA damage and the salivary secretion rates was observed.

8.1.2 Study II

In Study II, the effect of dietary supplementation with a sea buckthorn extract was studied in CKD patients. A crossover study (2 × 8 weeks, 4-weeks washout) was carried out with a patient group including 45 haemodialysis patients. The primary outcomes were DNA damage and oxidative DNA damage in the minor salivary glands. In addition, saliva production, inflammation and uremic markers were studied. The hypothesis was that the supplementation with sea buckthorn extract would have beneficial effects on the salivary glands in terms of DNA damage and oxidative DNA damage levels, as well as the saliva production. Our hypothesis was based on previous studies on sea buckthorn showing antioxidative effects, as well as beneficial effects on inflammation and mucous membranes[55,56,159].

The results from this intervention study showed that the sea buckthorn supplementation did not have significant effects on the levels of DNA damage or oxidative DNA damage in the salivary glands. The results are shown in **Table 5**. The median value of DNA damage was 5.2% DNA in tail before the supplementation, and 5.1% DNA in tail after the supplementation. The median value of oxidative DNA damage was 7.7% DNA in tail before the supplementation, and 8.8% DNA in tail after the supplementation.

Table 5. Effects of eight weeks of dietary supplementation with a sea buckthorn oil extract on DNA damage, saliva production, inflammation and blood markers in haemodialysis patients.

	Sea buckthorn supplementation						
	Before			After			
	Mean	Median	(Min - Max)	Mean	Median	(Min - Max)	p
DNA damage							
DNA damage (% DNA in tail)	5.5	5.2	(3.4 - 9.4)	5.3	5.1	(3.7 - 8.4)	0.23 [a]
Oxidative DNA damage (% DNA in tail)	9.1	7.7	(6.4 - 43.5)	8.9	8.8	(5.4 - 13.6)	0.12 [a]
Saliva production							
Secretion rate, at rest (mL/min)	0.1	0.1	(0.0 - 0.6)	0.1	0.0	(0.0 - 0.8)	0.29 [a]
Secretion rate, stimulated (mL/min)	1.0	0.9	(0.0 - 2.5)	1.0	0.9	(0.0 - 3.0)	0.46 [b]
Inflammation markers							
C-reactive protein (hsCRP) (mg/L)	6.7	4.2	(0.2 - 65.0)	9.4	5.4	(0.4 - 77.2)	0.24 [a]
Antitrypsin (g/L)	1.5	1.4	(0.9 - 2.3)	1.5	1.5	(0.6 - 2.4)	0.48 [b]
Orosomucoid (g/L)	1.0	1.0	(0.6 - 2.1)	1.0	1.0	(0.6 - 1.8)	0.55 [a]
Leukocytes (10^9/L)	7.2	6.8	(1.8 - 14.6)	7.1	6.9	(2.8 - 11.2)	0.93 [a]
Blood markers							
Albumin (g/L)	34.3	35.0	(21.0 - 44.0)	34.3	34.0	(25.0 - 44.0)	0.68 [a]
Calcium (Albumin corrected) (mmol/L)	2.4	2.5	(1.9 - 1.9)	2.4	2.4	(1.7 - 3.1)	0.43 [b]
Phosphate (mmol/L)	1.5	1.5	(0.8 - 2.5)	1.7	1.7	(0.9 - 2.7)	* [b]
Potassium (mmol/L)	5.1	5.2	(2.8 - 6.9)	5.0	4.9	(3.2 - 9.6)	0.36 [a]
Sodium (mmol/L)	138	137	(132 - 146)	139	138	(132 - 146)	* [a]
Creatinine (µmol/L)	746	690	(345 - 1293)	761	765	(384 - 1308)	0.39 [b]
Urea (mmol/L)	22.2	21.2	(6.0 - 47.8)	22.9	22.8	(8.9 - 39.7)	0.40 [a]
Carbon dioxide (mmol/L)	24.3	24.0	(15.0 - 32.0)	23.9	24.0	(19.0 - 30.0)	0.39 [a]
Glucose (mmol/L)	5.9	5.4	(4.1 - 11.0)	5.9	5.4	(2.2 - 11.0)	0.99 [a]
Haptoglobin (g/L)	1.1	1.0	(0.1 - 2.9)	1.2	1.1	(0.1 - 2.5)	0.30 [b]
Hemoglobin (g/L)	120	120	(95 - 156)	120	122	(89 - 145)	0.82 [b]
Thrombocytes (10^9/L)	218	214	(54 - 374)	221	212	(70 - 389)	0.61 [b]
Transferrin (g/L)	1.8	1.8	(0.8 - 2.7)	1.8	1.8	(1.0 - 2.6)	0.87 [b]
Immunoglobulin A (g/L)	2.6	2.5	(0.1 - 6.4)	2.7	2.4	(0.1 - 6.3)	0.46 [a]
Immunoglobulin G (g/L)	11.3	11.0	(4.8 - 21.9)	11.6	10.8	(5.5 - 24.1)	0.13 [a]
Immunoglobulin M (g/L)	0.7	0.7	(0.1 - 3.5)	0.8	0.7	(0.2 - 3.8)	0.21 [a]
Iron (µmol/L)	12.0	11.0	(4.0 - 32.0)	10.7	10.0	(5.0 - 25.0)	* [a]
Iron saturation	0.3	0.2	(0.1 - 0.7)	0.2	0.2	(0.1 - 0.6)	0.05 [a]

a) Non-normal distribution
b) Normal distribution
*p<0.05

A few *in vitro* studies have previously investigated the effect of sea buckthorn extracts on DNA damage. Geetha *et al.*[56] showed that, measured by the comet assay, flavones extracted from sea buckthorn decreased the levels of DNA damage induced by *tert*-butyl hydroperoxide in isolated rat lymphocytes. Moreover, radioprotective effects of a sea buckthorn extract containing high levels of polyphenols were shown by Shukla *et al.*[160] in murine thymocytes. An alkaline halo assay, similar to the comet assay, was used and scavenging of ROS induced by the radiation was shown and suggested to contribute to the radioprotective effects.

One explanation to the lack of an antioxidative effect on the DNA damage in the present study could be that the initial levels of DNA damage actually were lower than expected, as observed in Study I. It is possible that analysis of PBMC, in which previous studies have shown elevated levels of DNA damage, would have shown different results compared to what was observed in the saliva glands.

No significant effects were observed on the saliva production rates, inflammation markers and creatinine or urea levels on the supplementation of sea buckthorn. While Larmo *et al.*[8] showed a significant effect of sea buckthorn on CRP levels (median reduction was -0.059 mg/L), no significant changes in the inflammation markers were seen in the present study. The conflicting results may be a result of differences

in the supplement content; in the study by Larmo *et al.,* sea buckthorn puree, with higher content of water-soluble compounds was supplemented. In another study by Larmo *et al.*[9], an attenuation of increased tear film osmolarity was detected after administration of sea buckthorn. Even though no changes in the fatty acid content of the tear film were detected, a mechanism involving modulation of the local inflammation status of dry eyes was proposed.

Although the differences were small, the phosphate and sodium levels significantly increased after sea buckthorn supplementation, from 1.5 and 137 mmol/L to 1.7 and 138 mmol/L, respectively, after the supplementation ($p=0.02$ for both phosphate and sodium). Sodium is an important osmolyte where alterations in the serum levels of sodium could have an impact on osmosis and cellular functions. Increased phosphate levels are also potentially harmful as hyperphosphatemia stimulates the vascular calcification, a risk factor for developing atherosclerosis and cardiovascular disease[161]. Iron was significantly ($p=0.05$) reduced after supplementation, from 11.0 to 10.0 mmol/L. Since iron is important for oxygen transport, and iron deficiency can lead to anaemia, the observed reduced effect is potentially harmful. However, the changes were small and may not be of clinical relevance.

Dietary supplementation with sea buckthorn has not been studied in CKD patients previously. However, there are a few studies that have investigated other fruit and berry juice or extracts; one example is a recently presented crossover study on the effects of pomegranate supplementation on oxidative stress, inflammation and serum lipids in haemodialysis patients. Neither the biomarkers for oxidative stress (plasma F2 isoprostanes and isofuranes), the inflammation markers (CRP and IL-6), nor lipid profiles were affected[162]. On the contrary, a study by Shema-Didi *et al.*[163] showed that one year of intake of pomegranate juice had positive effects in haemodialysis patients, with ameliorated levels of investigated biomarkers for oxidative stress and inflammation (including oxidation protein products, malondialdehyde, IL-6 and TNF-α) compared to the placebo juice.

In Study II, the placebo treatment did in fact affect some of the measured parameters, as seen in **Table 6**. There was a significant increase in creatinine ($p=0.030$) and urea ($p=0.009$) levels, as well as immunoglobulin A ($p=0.04$) and M ($p=0.01$), while the CO_2-level decreased after the placebo treatment ($p=0.004$). The placebo capsules contained coconut oil, which is a commonly used placebo oil in studies on fatty acid supplementation. Since creatinine and urea accumulate in blood when kidney function is impaired, these findings suggest that a different placebo substance should be used in future intervention studies in CKD patients.

Table 6. Effects of eight weeks placebo treatment on DNA damage, saliva production, inflammation and blood markers in haemodialysis patients.

	Placebo treatment						
	Before			After			
	Mean	Median	(Min - Max)	Mean	Median	(Min - Max)	p
DNA damage							
DNA damage (% DNA in tail)	5.5	5.2	(3.6 - 13.2)	5.9	5.3	(3.6 - 11.5)	0.20 [a]
Oxidative DNA damage (% DNA in tail)	9.0	8.8	(4.6 - 24.2)	8.6	8.2	(4.1 - 13.7)	0.89 [b]
Saliva production							
Secretion rate, at rest (mL/min)	0.1	0.1	(0.0 - 0.5)	0.1	0.1	(0.0 - 0.9)	0.23 [a]
Secretion rate, stimulated (mL/min)	1.1	1.1	(0.0 - 2.4)	1.1	0.9	(0.0 - 3.3)	0.97 [a]
Inflammation markers							
C-reactive protein (hsCRP) (mg/L)	10.1	5.0	(0.4 - 77.0)	9.0	4.5	(0.2 - 95.9)	0.63 [a]
Antitrypsin (g/L)	1.5	1.6	(0.8 - 2.1)	1.5	1.5	(0.7 - 2.1)	0.37 [b]
Orosomucoid (g/L)	1.1	1.0	(0.4 - 2.0)	1.0	1.0	(0.6 - 1.6)	0.31 [a]
Leukocytes (10^9/L)	7.1	7.1	(2.6 - 11.0)	7.1	6.8	(3.3 - 12.6)	0.82 [b]
Blood markers							
Albumin (g/L)	34.4	34.0	(22.0 - 43.0)	34.6	35.0	(25.0 - 41.0)	0.60 [b]
Calcium (Albumin corrected) (mmol/L)	2.4	2.5	(2.1 - 2.9)	2.5	2.5	(2.2 - 2.7)	0.43 [b]
Phosphate (mmol/L)	1.7	1.6	(0.9 - 3.2)	1.8	1.8	(0.9 - 3.1)	0.46 [a]
Potassium (mmol/L)	5.0	4.9	(3.1 - 6.6)	5.4	5.4	(3.7 - 8.4)	* [b]
Sodium (mmol/L)	138	138	(131 - 150)	138	138	(128 - 143)	0.54 [b]
Creatinine (µmol/L)	719	693	(346 - 1078)	774	751	(400 - 1402)	* [b]
Urea (mmol/L)	21.8	22.0	(8.2 - 40.0)	24.5	24.6	(10.9 - 54.3)	** [a]
Carbon dioxide (mmol/L)	24.4	24.0	(20.0 - 29.0)	23.1	24.0	(14.0 - 29.0)	** [a]
Glucose (mmol/L)	6.0	5.4	(4.2 - 11.0)	6.3	5.2	(3.9 - 20.2)	0.40 [a]
Haptoglobin (g/L)	1.3	1.2	(0.1 - 2.7)	1.2	1.2	(0.1 - 2.7)	0.61 [b]
Hemoglobin (g/L)	120	121	(94 - 149)	123	123	(91 - 159)	0.27 [b]
Thrombocytes (10^9/L)	213	189	(59 - 343)	211	204	(106 - 395)	0.81 [a]
Transferrin (g/L)	1.8	1.8	(1.1 - 2.6)	1.8	1.8	(1.1 - 2.9)	0.15 [b]
Immunoglobulin A (g/L)	2.7	2.6	(0.1 - 5.6)	2.8	2.5	(0.1 - 6.3)	* [b]
Immunoglobulin G (g/L)	11.2	10.7	(4.9 - 22.0)	11.4	10.9	(5.4 - 5.4)	0.33 [b]
Immunoglobulin M (g/L)	0.7	0.7	(0.1 - 3.2)	0.8	0.7	(0.1 - 3.6)	** [a]
Iron (µmol/L)	12.2	11.0	(5.0 - 38.0)	11.4	10.0	(7.0 - 22.0)	0.74 [a]
Iron saturation	0.3	0.2	(0.1 - 0.7)	0.3	0.2	(0.1 - 0.6)	0.73 [a]

a) Non-normal distribution
b) Normal distribution
*p<0.05, **p<0.01

The study design might also have had an impact on the results. A crossover study has the advantage of acquiring fewer study subjects since the patients are their own controls. This design also decreases the influence of variation in the disease state of the patient and covariates. The design is based on the assumptions that the disease condition is stable during the study period, and that the period is short enough to avoid period effects such as seasonal variations. In addition, the effect of the intervention should not be permanent and the wash-out period between the treatment periods should be long enough to prevent carry-over effects. Violations of the assumptions may result in incorrect data analysis. In the present study, the kidney disease was assumed to remain stable during the study period of total 20 weeks. However, period effects of some parameters, including CO_2, potassium, calcium-phosphate product and calcium levels were observed. Further, despite a wash-out period of four weeks, a carry-over effect on iron levels was found. However, the statistical analysis carried out in this thesis was not corrected for multiple comparisons. If adjustments on p-values had been carried out, a majority of the period effects had not been statistically significant. Crossover studies are also more sensitive to drop-outs compared to parallel study designs since paired analysis is performed. The study in this thesis had 16 drop-outs, due to acute illness, non-compliance and deaths, which limited the output of the study. It should also be noted

that sea buckthorn is rather sour, which can result in increased study dropouts. In a study by Eccleston *et al.*[164], 33% of the study participants dropped-out due to gastro-intestinal intolerance of the sea buckthorn and the placebo juice. A majority of the participants completing that study reported the addition of sweetener to improve the taste. No significant effects on risk factors for coronary heart disease were observed[164].

A limitation of Study I was the lack of measurement of the actual levels of the supplemented nutrients (the fatty acids and the vitamin A and E) in the study objects. Both baseline values as well as levels after treatment would have been valuable information for interpreting the study results.

Even though some of the previously published studies on sea buckthorn were *in vivo* studies, the majority of the previous findings on antioxidative effects were performed in cultured cells. The present results point out the limitations in extrapolating *in vitro* findings to *in vivo* systems. As stressed by Halliwell[165], cultured cells are in many ways different from cells *in vivo.* Cultured cells are often cancer cells and divide more rapidly compared to non-cancerous cells. Cultured cells are also exposed to higher levels of oxidative stress, caused by increased ROS due to elevated oxygen tension, and due to possible lower levels of antioxidants such as vitamin C and E in the cell culture medium[165]. In addition, some cell culture media contain added salts of transition metals, of which the metal ions can generate ROS. Studies on oxidative and antioxidative effects in cultured cells must therefore be carefully designed and interpreted.

Study II further points out the contradictions in dietary supplements versus dietary intake of fruits and vegetables. The dietary antioxidants are thought to protect the biomolecules from oxidative damage, and thereby lower the risk for disease. Focusing on the damage to the DNA, the antioxidative protection is thought to lower the risk for cancer. However, taking in to account the results from Study I and Study II, and previous studies on dietary supplementation, it is reasonable to conclude that the endogenous antioxidative systems play a more significant role in preventing/repairing damage to cellular molecules compared to added antioxidants in supplements.

In conclusion, dietary supplementation with a sea buckthorn extract did not affect the levels of DNA damage and oxidative DNA damage in the minor salivary glands of haemodialysis patients. No improvements of saliva production, inflammation or uremic markers were observed.

8.2 STUDY III AND IV: TOXICITY AND BIOCOMPATIBILITY OF NANOPARTICLES

The aims of the second part of this thesis were to identify particularly toxic, as well as biocompatible, nanoparticles and to further examine toxic mechanisms and possible applications.

8.2.1 Particle screening

The aim of this pre-study was to screen a range of different nanoparticles for their cytotoxicity and to further compare the cytotoxicity of a selection of nanoparticles in different cell types. Initially, 37 different nano- and microsized particles with varying composition were screened for their cytotoxicity towards A549 lung cells. The results showed that certain nanoparticles were more toxic compared to others, while several nanoparticles did not induce any cytotoxicity, see **Figure 13.** The results demonstrated that the chemical composition is important in nanotoxicology and that all nanoparticles are not necessarily cytotoxic. However, it should be noted that the screening was performed using one concentration at one exposure time point and in one cell line. The Cu-based particles, especially the nanosized particles, showed particularly high cytotoxicity. Zn-based nanoparticles did also show cytotoxicity, which was further explored in the HL60 cell line in a study not included in this thesis. Silicon dioxide (SiO_2), also known as silica, and iron-based nanoparticles showed low cytotoxicity, thus, both particle types are attractive for biomedical applications. Owing to the feasibility of surface modifications and their biocompatibility, silica nanoparticles are used for example in implants, surface coatings and as delivery vectors for drugs or genes. The latter was further explored in Study IV. Iron oxide nanoparticles with superparamagnetic properties hold great promises for applications including contrast agents in magnetic resonance imaging (MRI), drug/gene delivery, multimodal imaging and biosensors[166]. However, conflicting results on the toxicity of both iron oxide nanoparticles[167] and silica nanoparticles[168] have been published. Therefore, caution should be taken when using these particles in applications with potential human exposure.

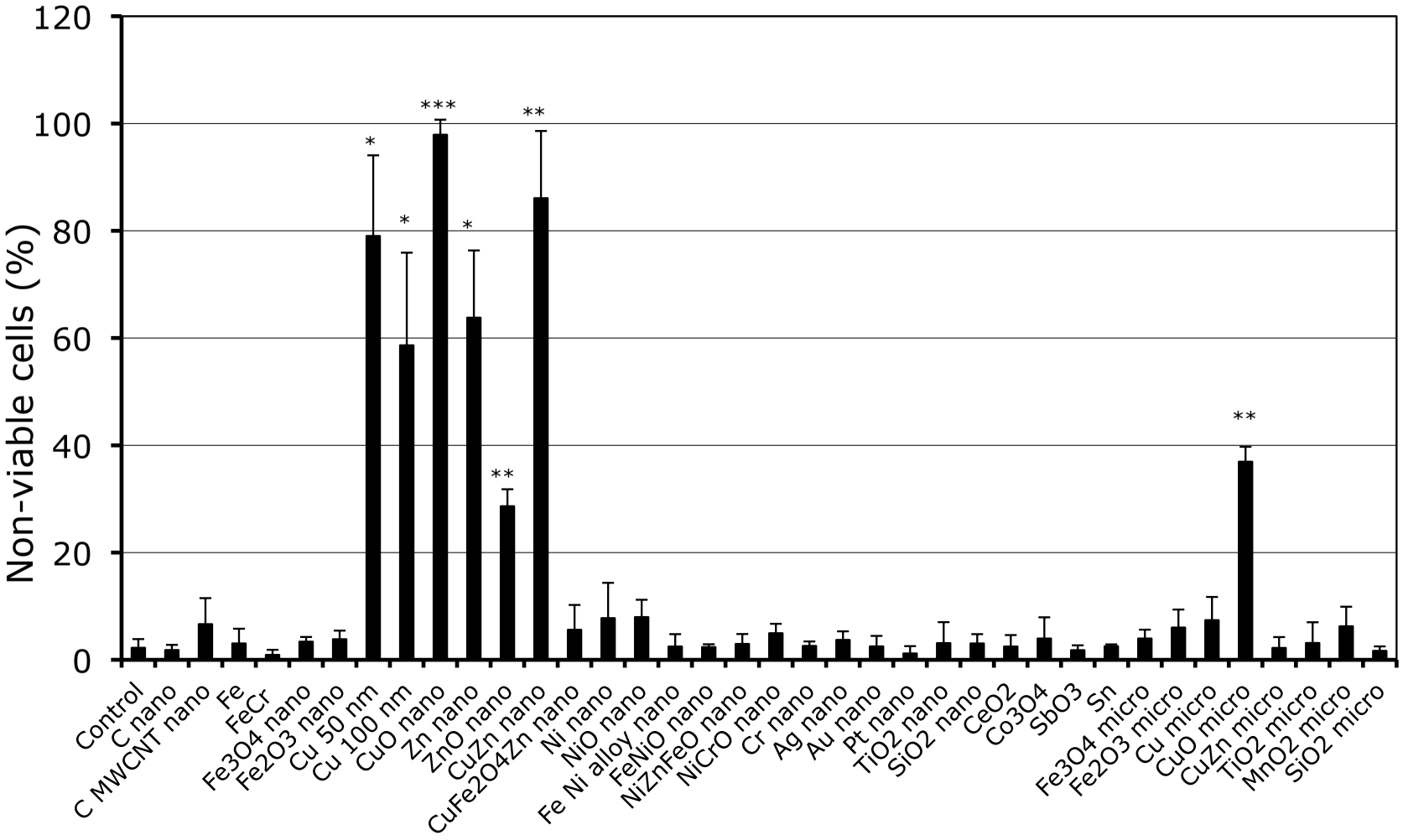

Figure 13. Cytotoxicity of 37 nano- and microparticles in A549 cells, measured by trypan blue staining after 18 h exposure to a particle concentration of 80 μg/mL. Unpaired two-tailed Student's t-test was performed to compare significant differences between each particle exposure and control. * represents $p<0.05$, **$p<0.01$ and ***$p<0.001$, respectively.

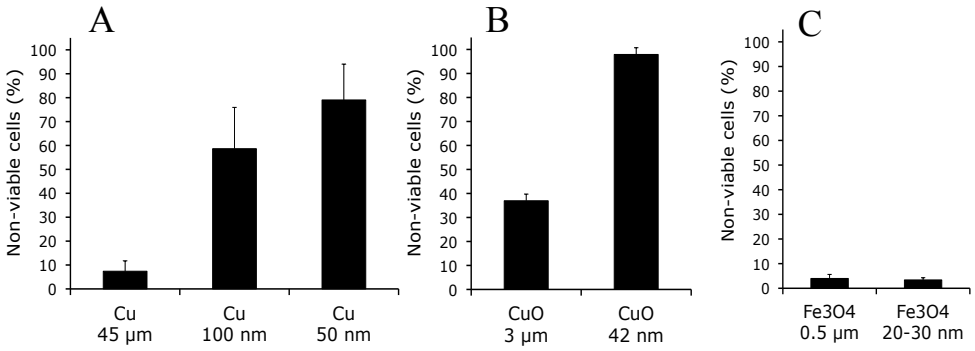

Figure 14. Comparison of size-dependent cytotoxicity of particles of Cu, CuO and Fe_3O_4 after exposing A549 cells to particle concentrations of 80 µg/mL for 18 h. Evaluated by trypan blue staining and one-way ANOVA analysis (followed by Tukey HSD post hoc test for the Cu particles). The size information was retrieved from to the manufacturers of the particles. **A**) Size-dependent effect ($p=0.002$) of three Cu particles with the sizes 45 µm, 100 nm and 50 nm respectively. **B**) Size-dependent effect ($p<0.001$) of two CuO particles with the sizes 3 µm and 42 nm. **C**) No observed size-dependent effect of Fe_3O_4 particles.

Size-dependent effects were observed for both Cu and CuO particles, with the smaller particles being more cytotoxic, as seen in **Figure 14**. The results are in agreement with previous studies showing that CuO nanoparticles are more toxic compared to microsized CuO particles in A549 cells[169,170]. Increased cytotoxicity and increased levels of DNA damage induced by the CuO nanoparticles were presented in these studies. The observed enhanced toxicity of nanoparticles compared to microparticles is often explained by a larger surface area and a higher surface reactivity. However, as can also be seen in **Figure 13** and **Figure 14**, no apparent differences in toxicity between nano- and microsized particles of Fe_3O_4, Fe_2O_3, TiO_2 and SiO_2 were observed.

After screening for particle toxicity, the next step was to investigate different cell types in their susceptibility towards nanoparticles. A selection of nanoparticles was investigated in six different cell types (A549, K562, HL60, Jurkat, PC-3 and isolated lymphocytes). One key finding was the great variety in the toxic response among the different cell types after particle exposure, as shown in **Figure 15**. The response differences were most pronounced for CuO, ZnO and Ag nanoparticles. Interestingly, the Ag nanoparticles were more toxic towards the isolated lymphocytes compared to the transformed cells of the different cell lines. This is alarming since Ag nanoparticles are widely used in many consumer products, e.g. for anti-odour effects in sports clothing. A review on biological effects on Ag nanoparticles was recently presented by Bartlomiejczyj *et al.*[171]. The toxicity of Ag nanoparticles was not further investigated in this thesis. The cell lines and the lymphocytes clearly differ from each other, since the cell lines are transformed cells and have therefore different cell signalling and cell death mechanisms compared to normal cells. In addition, varying mechanisms for cellular uptake, internalisation and intracellular fate of particles, depending on the cell type, can also influence the toxicity[132]. The importance of cell type selection was previously stressed by Bregoli *et al.*[172] who investigated the toxicity of Sb_2O_3 nanoparticles in hematopoietic progenitor cells from healthy donors

as well as in seven different hematopoietic cell lines. The authors reported that Sb_2O_3 nanoparticles inhibited the proliferation of erythroid progenitors but not granulocytic-monocytic progenitors from healthy donors. In addition, while the nanoparticles showed no adverse effects on proliferation in the cell lines, the differentiation of THP-1 cells (monocytic leukaemia cells) into macrophages was inhibited, which led to cell death. In this thesis, both lymphocytic Jurkat cells (T-lymphoblastic cells) and myelocytic cells (HL60 cells that are acute myeloid leukaemia cells and K562 cells that are chronic myeloid leukaemia cells) were studied. The differences in the toxic response between these cell lines were small. However, particularly upon exposure to CuO nanoparticles, certain differences became more prominent. A549 and PC-3 cells were shown to be more sensitive compared to the other cell types. Both A549 and PC-3 are epithelial cells, and they are adherent cells whereas HL60, K562 and Jurkat cells grow in suspension, which potentially affects the delivered dose to the cells and hence the toxic response. Differences among cell types in cellular uptake mechanisms or metabolic activity, may also explain the diversity in the toxic response, as discussed by Lanone *et al.*[173] and Chang *et al.*[174]. Differences in the contents of different cell media used for the adherent cells (DMEM) and the suspensions cells (RPMI) may also influence the toxicity. Therefore, these findings highlight the importance of the appropriate choice of cell type for nanotoxicology investigations.

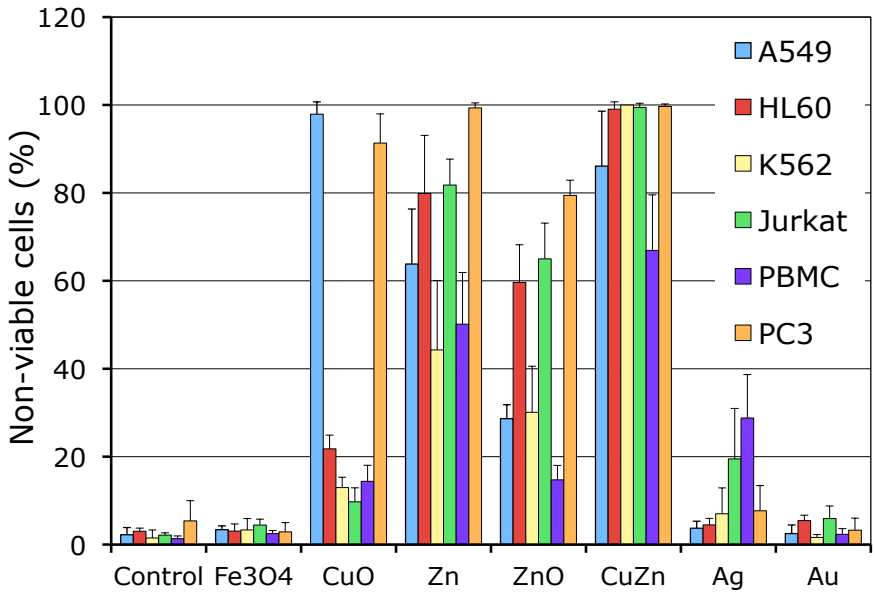

Figure 15. Cytotoxicity of seven selected nanoparticles in five different cell lines, and PBMC isolated from healthy blood donors. Measured by trypan blue staining after 18 h exposure to particle concentrations of 80 μg/mL.

8.2.2 Study III

In Study III, the mechanisms of the toxicity of Cu-based nanoparticles were further investigated in the myeloid HL60 cell line. Even though Cu is an essential trace element, excess levels may disturb the Cu homeostasis and lead to ROS formation via Fenton reactions, or dysfunctional enzymes[175]. Previous toxicity studies on Cu-based nanoparticles have mostly focused on epithelial cells. Since it has been shown that nanoparticles can translocate to the circulatory system, the HL60 cells were applied as a model for blood cells. The study focused on the effects of Cu and CuO nanoparticles on the cell viability, mitochondrial damage, DNA damage and oxidative DNA damage as well as the formation of ROS. As previous studies have proposed that the toxicity of CuO nanoparticles is caused by extra- or intracellular dissolution, toxic effects of dissolved Cu from easily soluble $CuCl_2$ were also investigated. In addition, particle characteristics including size, agglomeration and dissolution were investigated.

The results showed that both Cu and CuO nanoparticles were cytotoxic in the HL60 cells. The Cu nanoparticles showed high cytotoxicity at considerably lower concentrations compared to the CuO nanoparticles. Dose-response relationships were investigated and showed that the Cu nanoparticles were most toxic, followed by the Cu ions and lastly the CuO nanoparticles, as seen in **Figure 16**. The observed low toxicity of the CuO nanoparticles compared with the Cu ions differs from previous findings, in where CuO nanoparticles have been shown to be far more toxic in comparison with Cu ions[135,136,169]. In certain studies, the higher toxicity of CuO nanoparticles compared to Cu ions has been explained by a Trojan horse-type mechanism[135,136]. That is, the particulate form of CuO enables cellular uptake via endocytosis, and particle dissolution inside the cell can give rise to increased levels of Cu ions. The entrance of free ions from the extracellular environment into cells is normally regulated by metal sensing and transport proteins[134].

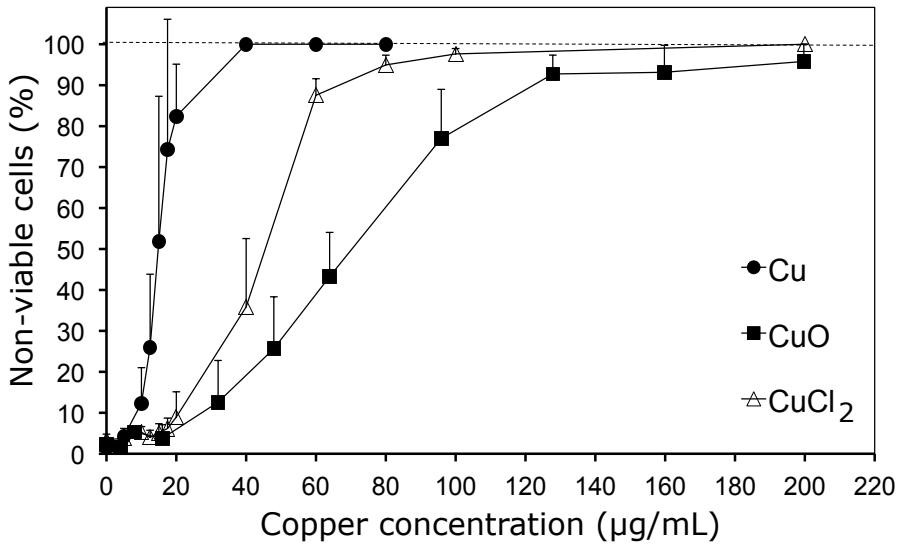

Figure 16. Dose-response relationships of Cu and CuO nanoparticles as well as $CuCl_2$ at equivalent copper concentrations in HL60 cells after 18 h exposure, evaluated by trypan blue staining, n=3.

The results in the present study demonstrated that HL60 cells are less sensitive to CuO nanoparticles compared to A549 cells, which could potentially be explained by differences in cellular uptake mechanisms. It is likely that A549 cells, which are epithelial cells, exert more endocytic activity compared to the HL60 cells. By comparing the toxicity of Cu, CuZn and CuO nanoparticles in A549 cells and HL60 cells, a greater difference in the toxic response between the two cell types was observed for the CuO nanoparticles in comparison with the Cu and CuZn nanoparticles, as shown in **Figure 17**. Karlsson *et al.*[176] showed that Cu and CuZn nanoparticles are more membrane reactive and induced more membrane damage compared to CuO nanoparticles. Hence it is possible that the toxicity of Cu nanoparticles is not as dependent on cellular uptake as the CuO nanoparticles, which can explain the lower variation in toxicity of Cu nanoparticles among different cell types. Further investigation on cellular uptake and cellular dose would nonetheless be necessary to support this theory. For instance, careful evaluation using endocytotic inhibitors such as chlorpromazine to study clathrin-mediated uptake, filipin and nystatin to study caveolae/lipid graft-mediated uptake or cytochalasin D to study actin-dependent uptake, could be conducted to obtain information on the cellular uptake mechanisms[177]. Furthermore, comparison of the particle characteristics in the different cell media used would be necessary.

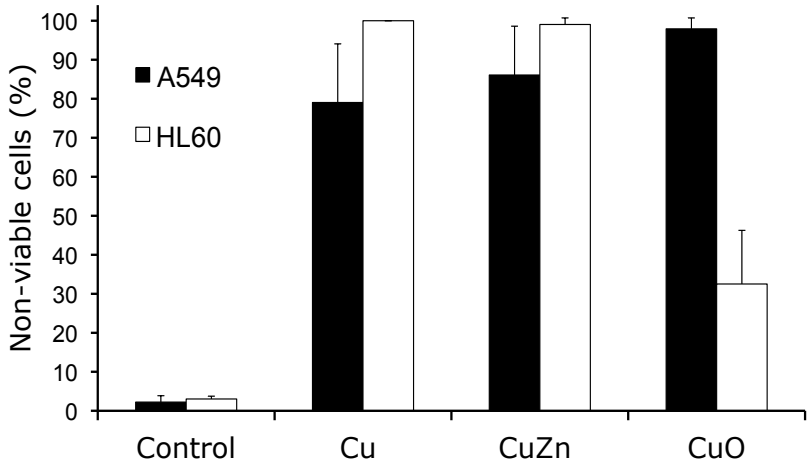

Figure 17. Comparison of cytotoxic effects of Cu, CuZn and CuO nanoparticles in A549 and HL60 cells, measured by trypan blue staining after 18 h exposure to particle concentration of 80 μg/mL, n=3.

Since oxidative stress has been proposed as one of the key toxicological mechanisms of nanoparticles, the oxidative effects of the Cu-based nanoparticles were evaluated using three strategies. The formation of ROS was initially studied in an acellular system in order to evaluate whether the nanoparticles were able to generate ROS intrinsically. Further, the intracellular ROS formation, as well as oxidative DNA damage, was studied in the HL60 cells. The results showed that the Cu nanoparticles induced high levels of ROS in the acellular setting. The intracellular levels of ROS induced by the Cu nanoparticles were slightly elevated, however the standard deviations were large. The DNA damage and the oxidative DNA damage showed a

tendency to be increased after exposure to Cu nanoparticles. While CuO nanoparticles did not induce acellular or intracellular ROS, or oxidative DNA damage, the Cu ions ($CuCl_2$) increased the acellular levels of ROS, but neither intracellular ROS nor oxidative DNA damage was observed. The results differ from previous findings where higher levels of intracellular ROS and oxidative stress have been reported upon exposure to CuO nanoparticles. For example, Siddiqui *et al.*[178] showed, after exposing HepG2 cells to CuO nanoparticles, a dose-dependent increase in lipid peroxidation and a dose-dependent decrease in glutathione levels. The authors also showed that N-acetyl-cystein protected against cell death, indicating oxidative stress-mediated cytotoxicity.

The effect of the Cu-based nanoparticles on the mitochondria was investigated using TMRM. Mitochondrial depolarisation was observed already after 2 h exposure to Cu nanoparticles, while the CuO nanoparticles and the Cu ion, did not induce any observed changes during the first 6 h of exposure. After a longer exposure time (24 h), all exposure concentrations induced mitochondrial depolarisation. These time-dependent effects may be explained by the fact that Cu nanoparticles react more rapidly with the cell membrane, whereas the toxicity of CuO nanoparticles may be more dependent on cellular uptake and subsequent dissolution, as discussed above.

As analysed by Annexin V-PI staining, the main type of cell death appeared to be necrosis for both Cu and CuO nanoparticles, as well as for $CuCl_2$. However, further investigations are required to confirm the cell death type as the analysis was only performed at one exposure time point (18 h). It cannot be excluded that apoptotic processes are involved at earlier stages of the exposure.

The differences in toxicity profiles of Cu and CuO nanoparticles can potentially be explained by the different chemical compositions and dissolution profiles of the materials. Cu nanoparticles are composed of a metallic Cu core, which is covered by a thin oxide surface of Cu_2O and CuO. CuO nanoparticles are instead homogenously composed of CuO[179]. This difference in the composition will influence the metal release processes from these particles; the release from the Cu nanoparticles is governed by chemical and electrochemical processes, whereas the release from the CuO nanoparticles is governed by chemical processes. It is thus possible, that the high levels of ROS induced by Cu nanoparticles in the acellular assay, in Study III, are due to the electrochemical and chemical dissolution of Cu nanoparticles. In these processes both Cu(I) and Cu(II) are released, which can take part in redox reactions. In a previous study Li *et al.*[180] proposed that the observed toxicity of Cu nanoparticles in *E.coli* was caused by the generation of H_2O_2, mediated by the release of Cu(I). The importance of the valence state of the released ions on the toxicity was stressed, and it was suggested that the toxicity of Cu nanoparticles was independent on Cu(II), explaining the observed lower toxicity of $CuCl_2$[180].

Investigation of the released amount of Cu ions into cell media using AAS, showed that the release was fast and started directly after the sonication of the Cu nanoparticles dispersions, whereas the release from the CuO nanoparticles was slower. This difference in metal release kinetics may, together to the difference in oxidation state of the released species, explain the higher cytotoxicity observed for

Cu nanoparticles compared to CuO nanoparticles. It is also likely that the release of Cu ions from the Cu nanoparticles is enhanced upon contact with the cells, since the cell membrane contains three times more oxygen compared to the cell medium, as discussed by VanWinkle *et al.*[181]. An interesting further investigation of this study would be to examine the released species from the particles, including evaluation of the oxidation state as well as the complexation products that are likely to be formed between the ions and cell medium constituents[182].

In addition to the intrinsic properties of nanoparticles, several methodological settings affect the outcome of studies on biological interactions of nanoparticles. This includes the preparation of the nanoparticles prior to the cell exposure, e.g. the sonication and the presence or absence of serum in the cell medium. Sonication, using an ultrasonic probe or bath, is a commonly applied procedure to disperse particles in cell medium prior cell exposure. Fetal bovine serum is often added to cell culture medium as growth factor supplementation. In a study by Cronholm *et al.*[183], it was shown that sonication increased the cytotoxicity of Cu nanoparticles, whereas the presence of serum in the cell culture media increased the Cu release, but did not affect the cytotoxicity. In fact, Kim *et al.*[184] showed that increased levels of serum suppressed the cytotoxicity induced by amine-modified polystyrene nanoparticles. Differences in the sonication procedures as well as the use of different serum content and levels may explain the contradictory results often reported from different studies.

Moreover, careful evaluation and relevant controls should be considered when choosing the assay for studying nanoparticle toxicity. Several nanoparticles have been shown to interfere with commonly used viability assays based on spectrophotometric analysis[185,186]. Interference with the resazurin assay was observed for ZnO nanoparticles in the present investigation. The trypan blue exclusion assay was therefore used to evaluate cell viability. The trypan blue-stained cells were counted manually under a light microscope and potential optical interferences are thus diminished. However, this method has the disadvantage of being rather time-consuming and less sensitive, as only the late stages of the cell death is detected[187]. It should also be noted that the disability of a cell to exclude the trypan blue dye does not necessarily mean that the cell is dead; it is rather a measure of the cell membrane integrity[188]. Thus, the results from the trypan blue assay were compared with the results from a second cell death analysis assay, the Annexin V-PI assay, where good correlation between the methods was observed for CuO nanoparticles, while a notable discrepancy was observed for the Cu nanoparticles. While the trypan blue assay showed a higher cytotoxicity of the Cu nanoparticles compared to the Annexin V-PI assay, underestimation of the cytotoxicity using the trypan blue assay would have been more probable, since the trypan blue molecule is larger compared to the PI molecules used in the Annexin V-PI assay and hence is more easily excluded by the cells.

The lack of evaluation of endotoxin contamination of the nanoparticles is a limitation of the study. Due to the reactive surface of nanoparticles, molecules including endotoxins can easily be adsorbed to the surface. Contamination of nanoparticles can give rise to misinterpretation of toxicological data, both *in vitro* and *in vivo*.

In conclusion, the results from the particle screening and Study III showed that Cu-based nanoparticles were cytotoxic. The toxic response varied among different cell types, potentially due to different uptake mechanisms. Cu nanoparticles revealed higher cytotoxicity compared to CuO nanoparticles and $CuCl_2$ in the HL60 cells. The Cu nanoparticles induced high oxidation in an acellular setting, as well as slightly increased levels of intracellular ROS and oxidative DNA damage. CuO nanoparticles did not induce acellular ROS and the intracellular ROS and DNA damage was limited. Differences in the metal release processes might explain the different toxicity profiles of the Cu and CuO nanoparticles.

8.2.3 Study IV

Silica nanoparticles offer several exciting opportunities for usage in nanomedicine, owing to the feasibility in designing the particles with a desired function, as well as their low intrinsic toxicity. For example, particles with porous structure can be produced, and the size and pore volume can be adjusted to increase the drug/gene loading capacity and to control cargo release processes[189]. In Study IV, nonporous and mesoporous forms of silica nanoparticles, modified with propylamine-groups, were investigated for their biocompatibility and ability to deliver plasmid DNA in MCF-7 breast carcinoma cells. Thorough investigation of the physicochemical properties, as well as the influence of serum in the cell medium was performed as these factors have been shown to strongly affect the interactions of nanomaterials with biological systems.

The surface charge of nanoparticles is an important physicochemical property that has a great impact on the biological interactions. Therefore, DLS measurements were performed to calculate the zeta potential, which provides information regarding the surface charge of the particles. It should however be noted that the zeta potential is affected by both the surface charge of the particles, as well as the solvent, which in this study was DMEM. Both the nonporous and the mesoporous amine-modified particles were shown to have a slight positive charge in DMEM (without added serum), with the latter showing less positive charge. According to the manufacturer, the particles have been modified with the same amount (wt%) of propylamine. The mesoporous particles should therefore have a lower amount of amine-groups on the external surface (not including the pores), which would explain the less positive charge compared to the nonporous particles. Delivery vectors are commonly cationic to facilitate the complexation of the particles with the negatively charged DNA, and to enable the following cellular internalisation. Positively charged particles attach more easily to the anionic hydrophilic cell membrane and are in general also taken up by cells more readily[190]. Consequently, cationic particles are generally also more cytotoxic compared to neutral or negatively charged particles[191]. A neutral charge of the particle and DNA complex is favourable for *in vivo* applications, in order to avoid interaction with blood components and prolong the blood circulation time[192]. Surface modifications with polymers such as PEG can be used to mask the particle charge and increase the hydrophilicity, and thereby prevent unspecific protein interactions[107]. In Study IV, the surface charge of the nonporous and the mesoporous particles became slightly negative upon DNA complexation. Cellular uptake was

studied by TEM and the silica nanoparticles were internalised in agglomerates, as shown in **Figure 18**.

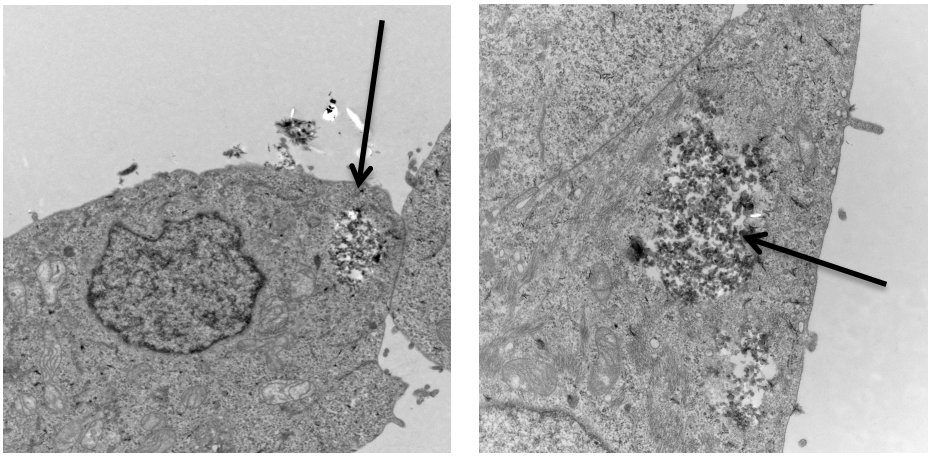

Figure 18. TEM images of MCF-7 cells exposed to mesoporous silica nanoparticles. Photo by Kjell Hultenby.

The delivery efficiency was higher for the nonporous silica nanoparticles compared to the mesoporous particles. This was observed both with and without serum in the cell medium. The delivery efficiency of both particles in the presence of serum is shown in **Figure 19**. The pore size of the mesoporous particles was relatively small (2.4 nm), which is a likely explanation of their poor delivery efficiency as the plasmid DNA was too large to be loaded in the pores. In general, particles with small pore size are suitable for encapsulating smaller drug molecules in the pores, potentially combined with larger biomolecules such as DNA and RNA, adsorbed or anchored on the external surface[193]. However, DNA is vulnerable to enzymatic degradation, which is a challenge to overcome for *in vivo* applications. This can be solved by using particles with larger-sized pores that are more suitable for gene encapsulation within the pores[194,195]. Kim *et al.*[194] showed that mesoporous silica nanoparticles with ultralarge pores (23 nm pore size) were superior in plasmid DNA delivery efficiency in HeLa cells, compared to those with small pores (2 nm pore size). Ultralarge pores also improved the delivery of small interfering RNA (siRNA), both *in vitro* and *in vivo*, compared to silica particles with smaller pores[195]. The siRNA was also protected from enzymatic degradation when the particles with ultralarge pores were used. Thus, increasing the pore size might be a possible approach to improve the delivery efficiency of silica nanoparticles.

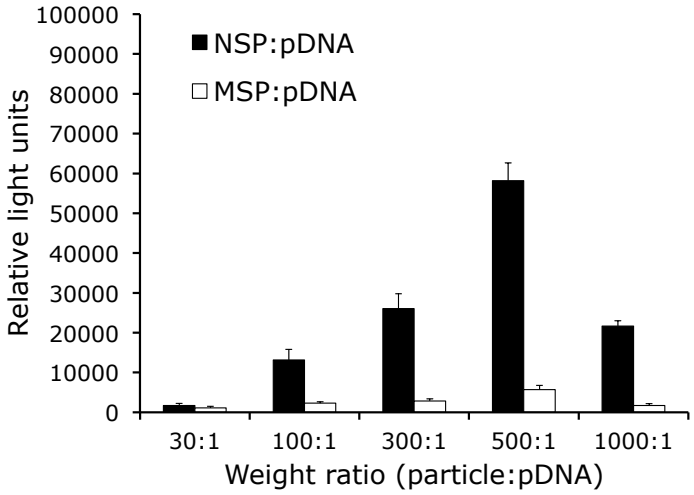

Figure 19. Delivery efficiency of nonporous (NSP) and mesoporous (MSP) silica nanoparticles complexed with luciferase-encoding plasmid DNA (pDNA) at different weight ratios. n=3.

The greater delivery efficiency of the nonporous compared to the mesoporous particles could, in parallel with the difference in surface charge of the particles, be explained by the higher external surface area of the nonporous particles. Functionalisation of the particles with propylamine groups to the same extent (wt%) would lead to a larger amount of amine-groups present at the external surface of the nonporous particles, as a considerable part of the amine groups is present within the pores of the mesoporous particles[196]. As a result, the actual binding of the DNA is decreased for mesoporous particles as is the interaction with the cell and the cellular uptake. As suggested by Slowing *et al.*[196], this smaller external surface area of porous silica particles can decrease their interaction with cell membranes of red blood cells and thereby reduce their haemolytic activity.

The delivery efficiency of the both tested types of silica nanoparticles was anyhow considerably lower compared to the commercially available *in vitro* transfection agent Lipofectamine® 2000, which gave a relative light unit (RLU)/cell viability of 1×10^6. Approaches to further increase the delivery efficiency of silica nanoparticles include functionalisation using polyethyleneimine (PEI)[197] and cell penetrating peptides[198]. Recently, Brevet *et al.*[106] reported improved plasmid DNA delivery efficiency *in vitro*, using histidine-functionalised silica nanoparticles in comparison to amine-modified silica nanoparticles. However, *in vivo*, both particles showed similar efficiencies.

Both of the studied amine-modified silica nanoparticles showed excellent biocompatibility, i.e. the toxicity was low. The biocompatibility of the complex of particle and plasmid DNA was studied using the MTT assay, and the biocompatibility of the silica nanoparticle vectors alone were studied using the CFE assay. The nonporous silica particles showed slightly higher toxicity compared to the mesoporous particles in the absence of serum in the cell culture medium, as shown in **Figure 20A** and **Figure 20C**. These findings are in line with previous studies, where nonporous silica particles were found to be more toxic compared to porous particles[196,199]. Interestingly, the toxicity of the porous silica particles was abolished

in the presence of serum, as shown in **Figure 20B** and **Figure 20D**. This is promising for *in vivo* applications as the presence of serum mimics better the conditions in biological fluids. Upon contact with biological fluids, or cell culture medium, proteins and other biomolecules will cover the surface of the nanoparticles, forming a protein corona[200]. The nature of the corona depends on the size and surface properties of the particle, but also on the composition of the solvent. The corona dictates at least the initial particle-cell interactions, including the cellular uptake and toxicity. It is generally considered that the protein corona leads to a decreased toxicity. However, its impact on the toxicity depends on the property of the particle. It has, for example, previously been shown that the protein corona on silica nanoparticles reduces their cytotoxicity and their haemolytic activity[148,201]. On the contrary, for other particles, such as ZnO and CuO nanoparticles, the presence of serum in the cell culture medium did not affect the toxicity to the same extent as for the silica nanoparticles[199,202]. This is potentially due to differences in the toxic mechanisms, which for ZnO and CuO nanoparticles are more dependent on the release of ions.

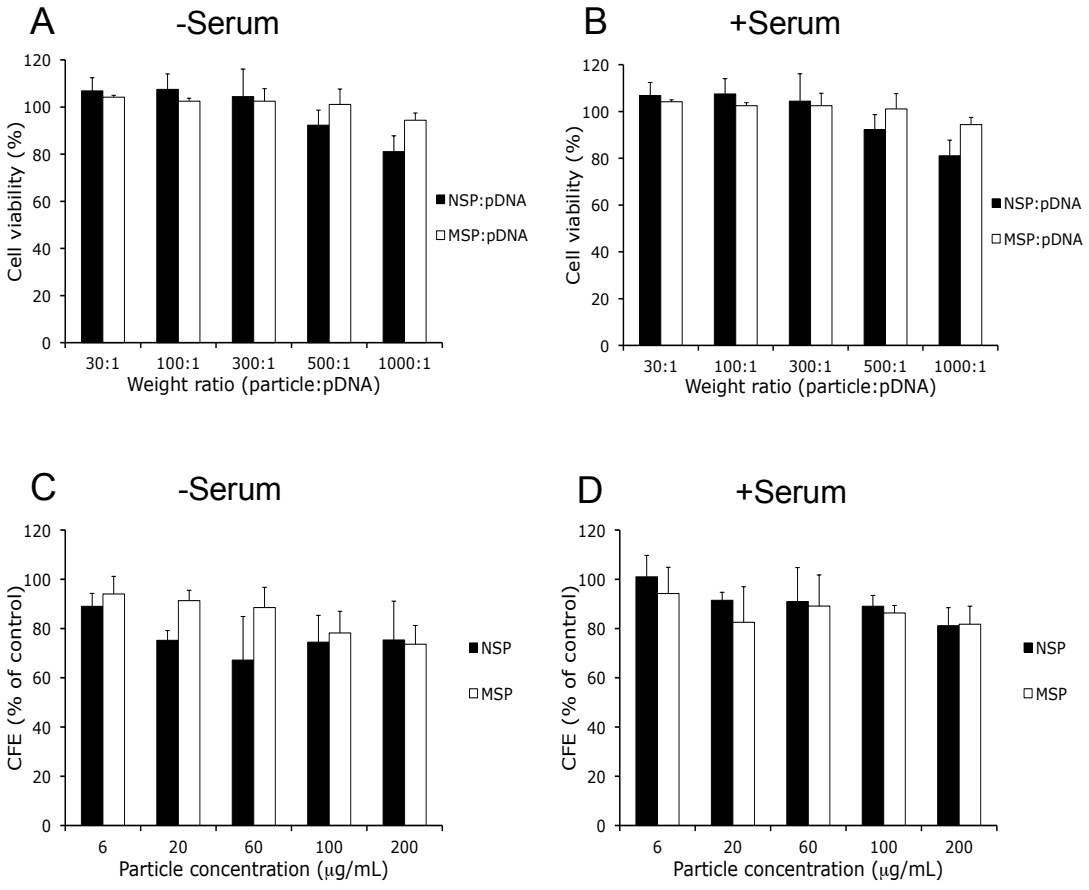

Figure 20. Biocompatibility of nonporous (NSP) and mesoporous (MSP) amine-modified silica nanoparticles in MCF-7 cells. **A)** Cell viability assessed by MTT analysis after exposure to NSP and MSP complexed with plasmid DNA (pDNA) in the absence of serum and **B)** in the presence of serum. **C)** CFE after exposure to NSP and MSP alone, in the absence of serum and **D)** in the presence of serum. n=3

In conclusion, the amine-modified silica nanoparticles showed good biocompatibility in the MCF-7 cells. The gene delivery efficiency was higher for the nonporous silica nanoparticles compared to the mesoporous particles. The presence of serum in the cell medium increased the delivery efficiency for the nonporous silica particles, as well as abolished the toxic effects for both particles. The results suggest that silica nanoparticles are promising as delivery vectors *in vivo*. However, further investigation is required to significantly improve the delivery efficiency as well as to understand the cellular uptake mechanisms.

9 SUMMARY AND CONCLUSIONS

Reactive oxygen species (ROS) are involved in several important redox reactions in cells and organisms. Concomitantly, ROS also have the potential to damage cellular macromolecules, potentially leading to disease development and ageing. A balance between the oxidative processes and antioxidative defence is necessary to allow the important cellular redox reactions to take place, and at the same time limit the oxidative damage to cellular macromolecules.

Chronic kidney disease (CKD) is associated with high levels of oxidative stress, as well as inflammation and malnutrition, which all contribute to the higher risk for cardiovascular disease in this patient group. In addition to the uraemia, factors including dialysis treatment, anaemia, medication, vitamin deficiency and diet, influence the oxidative stress level. Moreover, CKD patients also often suffer from impaired oral health. Higher levels of DNA damage have previously been reported in peripheral blood mononuclear cells (PBMC) in this patient group. However no previous studies on DNA damage and oxidative DNA damage in the salivary glands in this patient group have been found in the literature.

The aim of Study I was to investigate the levels of oxidative stress and DNA damage in minor salivary glands, as well as to assess the levels of saliva production, inflammation and uremic markers in CKD patients. In summary, the results showed that the levels of DNA damage in predialysis patients were higher compared to controls. Despite the observed increased levels of inflammation and uremic markers, the levels of DNA damage in the dialysis patients were lower compared to the controls. The oxidative DNA damage did not differ between the groups. As expected, the saliva production was lower in the CKD patients compared to the controls. The results suggest that peripheral tissue and the circulating blood cells are differently affected by kidney disease and dialysis, potentially due to upregulated antioxidative defence and DNA repair mechanisms induced by the dialysis treatment.

Supplementation with dietary vitamins and antioxidants is an attractive approach to decrease levels of oxidative stress and prevent disease. However, intervention studies have often failed in showing beneficial health effects of dietary supplementation, with some studies even demonstrating adverse health effects. The aim of Study II was to investigate the effects of a dietary supplement rich in antioxidants and fatty acids (a sea buckthorn extract) on DNA damage, oral health and inflammation in dialysis patients. The crossover study (2 × 8 weeks) showed that the supplementation did not have any effects on the DNA damage, oxidative DNA damage, inflammation or saliva production in the dialysis patients. The results add to the inconsistency and contradictions of the beneficial health effects of dietary supplementation.

Exposure to nanoparticles can potentially induce oxidative stress and adverse health effects. Nanoparticles exert properties attractive for several applications, e.g. in medicine, electronics and inks. Recent advances in nanotechnology bring on an increased usage and potentially increased human exposure. The health effects of exposure to nanoparticles are not sufficiently understood. Therefore, a further aim of

the thesis was to screen a range of particles for their cytotoxicity and to investigate the toxicity of selected nanoparticles in different cell types. In summary, Cu- and Zn-based nanoparticles were particularly cytotoxic and the toxicity varied among different cell types. Several nanoparticles, including iron oxide and silica nanoparticles, did not induce cytotoxicity. Size-dependent effects were observed for Cu and CuO particles, with the nanoparticles being more toxic compared to microsized particles.

The aim of Study III was to investigate the toxic mechanisms of Cu-based (Cu and CuO) nanoparticles in the human leukemic cell line HL60. In conclusion, Cu nanoparticles were more toxic compared to CuO nanoparticles and the Cu ions (from $CuCl_2$) at equivalent Cu mass doses. The Cu nanoparticles induced high oxidation in an acellular setting, as well as slightly increased levels of intracellular ROS and oxidative DNA damage. CuO nanoparticles did not induce acellular ROS and the intracellular ROS and DNA damage was limited. Mitochondrial damage was induced early in the exposure to Cu nanoparticles, whereas mitochondrial effects of CuO nanoparticles and $CuCl_2$ were observed in a later stage, indicating differences in toxicity modes. The main type of cell death appeared to be necrosis, however further investigations are needed to confirm the cell death type. Differences in the metal release processes may explain the different toxicity modes of the Cu and CuO nanoparticles.

The aim of Study IV was to study the biocompatibility of amine-modified silica nanoparticles and to examine their potential use as vectors for delivery of plasmid DNA in a human breast carcinoma cell line (MCF-7). In summary, the gene delivery efficiency was higher for the nonporous silica nanoparticles compared to the mesoporous particles. The presence of serum in the cell medium increased the delivery efficiency, as well as abolished the toxic effects. The results suggest that silica nanoparticles are promising as delivery vectors *in vivo*. However, further investigation is required to significantly improve the delivery efficiency as well as for increased understanding of the cellular uptake mechanisms.

9.1 OUTLOOK

The oxygen paradox, i.e. that aerobic cells are dependent on oxygen for life, yet oxygen is an inherently threat to the cells, is a challenging research field. Oxidative stress is associated with several diseases and also ageing. With the aim to decrease oxidative stress levels, the interest in dietary supplementation with antioxidants and vitamins is great. However, supplementation with antioxidants does seldom affect the levels of oxidative stress, as measured with different biomarkers. Further investigations on the relationship between endogenous antioxidants and repair systems upon increased levels of oxidative stress, caused by either disease or toxic agents in the environment, are needed for potential treatment possibilities. Potential risk groups or groups that could benefit from dietary supplement would also be appealing to identify.

Oxidative stress is also important early in life. New-borns, and especially pre-term delivered babies, are more sensitive to oxidative stress. The relationships between exposure to oxidative stress early in life and later consequences need further investigations. In an on-going collaboration between Karolinska Institutet and Karolinska University Hospital, we are investigating the impact of premature birth on the levels of oxidative stress and DNA damage in blood cells.

On account of the increased use of nanoparticles in society, there is a great need to understand the interactions of nanomaterials with biological systems. Increased knowledge of how nanoparticles interact with biological systems is a prerequisite for a safe development of nanoproducts including safe and efficient nanomedicine. Nanotoxicology and nanomedicine share several challenges and an increased understanding of how different physicochemical properties affect the biological fate is needed. Further studies on exposure to nanoparticles as well as biodistribution, cellular uptake and toxic mechanisms are required.

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11 REFERENCES

1. Halliwell B and Gutteridge J. *Free Radicals in Biology and Medicine.* 4th ed. Oxford, Oxford University Press, 2007.

2. Balaban RS, Nemoto S, Finkel T. Mitochondria, oxidants, and aging. *Cell.* 2005;120(4):483-495.

3. Bjelakovic G, Nikolova D, Gluud LL, Simonetti RG, Gluud C. Antioxidant supplements for prevention of mortality in healthy participants and patients with various diseases. *Cochrane Database Syst Rev.* 2012;3:CD007176.

4. Oberg BP, McMenamin E, Lucas FL, et al. Increased prevalence of oxidant stress and inflammation in patients with moderate to severe chronic kidney disease. *Kidney Int.* 2004;65(3):1009-1016.

5. Guzeldemir E, Toygar HU, Tasdelen B, Torun D. Oral health-related quality of life and periodontal health status in patients undergoing hemodialysis. *J Am Dent Assoc.* 2009;140(10):1283-1293.

6. Jha V, Garcia-Garcia G, Iseki K, et al. Chronic kidney disease: global dimension and perspectives. *Lancet.* 2013;382(9888):260-272.

7. Olsson ME, Gustavsson KE, Andersson S, Nilsson A, Duan RD. Inhibition of cancer cell proliferation in vitro by fruit and berry extracts and correlations with antioxidant levels. *J Agric Food Chem.* 2004;52(24):7264-7271.

8. Larmo P, Alin J, Salminen E, Kallio H, Tahvonen R. Effects of sea buckthorn berries on infections and inflammation: a double-blind, randomized, placebo-controlled trial. *Eur J Clin Nutr.* 2008;62(9):1123-1130.

9. Larmo PS, Jarvinen RL, Setala NL, et al. Oral sea buckthorn oil attenuates tear film osmolarity and symptoms in individuals with dry eye. *J Nutr.* 2010;140(8):1462-1468.

10. Rodhe Y, *Assessment of DNA damage, oxidative stress and inflammation in chronic kidney disease patients – and a clinical study of a dietary supplement.* Licentiate thesis. Karolinska Institutet. Sweden. 2012.

11. Krug HF, Wick P. Nanotoxicology: an interdisciplinary challenge. *Angew Chem Int Ed Engl.* 2011;50(6):1260-1278.

12. Oberdorster G, Oberdorster E, Oberdorster J. Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. *Environ Health Perspect.* 2005;113(7):823-839.

13. Inoue M, Sato EF, Nishikawa M, et al. Mitochondrial generation of reactive oxygen species and its role in aerobic life. *Curr Med Chem.* 2003;10(23):2495-2505.

14. Flint DH, Tuminello JF, Emptage MH. The inactivation of Fe-S cluster containing hydro-lyases by superoxide. *J Biol Chem.* 1993;268(30):22369-22376.

15. Brodie AE, Reed DJ. Reversible oxidation of glyceraldehyde 3-phosphate dehydrogenase thiols in human lung carcinoma cells by hydrogen peroxide. *Biochem Biophys Res Commun.* 1987;148(1):120-125.

16. Sies H. Oxidative stress: oxidants and antioxidants. *Exp Physiol.* 1997;82(2):291-295.

17. Sen CK, Packer L. Antioxidant and redox regulation of gene transcription. *Faseb J.* 1996;10(7):709-720.

18. The PyMOL Molecular Graphics System, Educational version 1.3.

19. Lindahl T. Instability and decay of the primary structure of DNA. *Nature.* 1993;362(6422):709-715.

20. Ciccia A, Elledge SJ. The DNA damage response: making it safe to play with knives. *Mol Cell.* 2010;40(2):179-204.

21. Norbury CJ, Hickson ID. Cellular responses to DNA damage. *Annu Rev Pharmacol Toxicol.* 2001;41:367-401.

22. Steenken S, Jovanovic S. How Easily Oxidizable Is DNA? One-Electron Reduction Potentials of Adenosine and Guanosine Radicals in Aqueous Solution. *J Am Chem Soc.* 1997;119:617-618.

23. Hwang ES, Bowen PE. DNA damage, a biomarker of carcinogenesis: its measurement and modulation by diet and environment. *Crit Rev Food Sci Nutr.* 2007;47(1):27-50.

24. Delaney S, Jarem DA, Volle CB, Yennie CJ. Chemical and biological consequences of oxidatively damaged guanine in DNA. *Free Radic Res.* 2012;46(4):420-441.

25. Valko M, Izakovic M, Mazur M, Rhodes CJ, Telser J. Role of oxygen radicals in DNA damage and cancer incidence. *Mol Cell Biochem.* 2004;266(1-2):37-56.

26. Valavanidis A, Vlachogianni T, Fiotakis C. 8-hydroxy-2' -deoxyguanosine (8-OHdG): A critical biomarker of oxidative stress and carcinogenesis. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev.* 2009;27(2):120-139.

27. Klungland A, Bjelland S. Oxidative damage to purines in DNA: role of mammalian Ogg1. *DNA Repair (Amst).* 2007;6(4):481-488.

28. Collins AR. The comet assay for DNA damage and repair: principles, applications, and limitations. *Mol Biotechnol.* 2004;26(3):249-261.

29. Sancar A, Lindsey-Boltz LA, Unsal-Kacmaz K, Linn S. Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. *Annu Rev Biochem.* 2004;73:39-85.

30. Indo HP, Yen HC, Nakanishi I, et al. A mitochondrial superoxide theory for oxidative stress diseases and aging. *J Clin Biochem Nutr.* 2015;56(1):1-7.

31. World Cancer Research Fund / American Institute for Cancer Research. Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective. Washington DC: AIR, 2007.

32. Ross AC, Zolfaghari R, Weisz J. Vitamin A: recent advances in the biotransformation, transport, and metabolism of retinoids. *Curr Opin Gastroenterol.* 2001;17(2):184-192.

33. The National Food Agency. http://www.livsmedelsverket.se/livsmedel-och-innehall/naringsamne/vitaminer-och-antioxidanter/ [Cited 2015-04].

34. Young AJ, Lowe GM. Antioxidant and prooxidant properties of carotenoids. *Arch Biochem Biophys.* 2001;385(1):20-27.

35. Levine M, Rumsey SC, Daruwala R, Park JB, Wang Y. Criteria and recommendations for vitamin C intake. *Jama.* 1999;281(15):1415-1423.

36. Duarte TL, Lunec J. Review: When is an antioxidant not an antioxidant? A review of novel actions and reactions of vitamin C. *Free Radic Res.* 2005;39(7):671-686.

37. Lane DJ, Richardson DR. The active role of vitamin C in mammalian iron metabolism: much more than just enhanced iron absorption! *Free Radic Biol Med.* 2014;75:69-83.

38. Clement MV, Ramalingam J, Long LH, Halliwell B. The in vitro cytotoxicity of ascorbate depends on the culture medium used to perform the assay and involves hydrogen peroxide. *Antioxid Redox Signal.* 2001;3(1):157-163.

39. Martin BD, Schoenhard JA, Hwang JM, Sugden KD. Ascorbate is a pro-oxidant in chromium-treated human lung cells. *Mutat Res.* 2006;610(1-2):74-84.

40. Bergstrom T, Ersson C, Bergman J, Moller L. Vitamins at physiological levels cause oxidation to the DNA nucleoside deoxyguanosine and to DNA--alone or in synergism with metals. *Mutagenesis.* 2012;27(4):511-517.

41. Halliwell B. Vitamin C and genomic stability. *Mutat Res.* 2001;475(1-2):29-35.

42. Traber MG. Vitamin E regulatory mechanisms. *Annu Rev Nutr.* 2007;27:347-362.

43. Ahsan H, Ahad A, Iqbal J, Siddiqui WA. Pharmacological potential of tocotrienols: a review. *Nutr Metab (Lond).* 2014;11(1):52.

44. Wang X, Quinn PJ. Vitamin E and its function in membranes. *Prog Lipid Res.* 1999;38(4):309-336.

45. Block G, Patterson B, Subar A. Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. *Nutr Cancer.* 1992;18(1):1-29.

46. Joshipura KJ, Hu FB, Manson JE, et al. The effect of fruit and vegetable intake on risk for coronary heart disease. *Ann Intern Med.* 2001;134(12):1106-1114.

47. Ivey KL, Hodgson JM, Croft KD, Lewis JR, Prince RL. Flavonoid intake and all-cause mortality. *Am J Clin Nutr.* 2015;101(5):1012-1020.

48. Bellavia A, Larsson SC, Bottai M, Wolk A, Orsini N. Fruit and vegetable consumption and all-cause mortality: a dose-response analysis. *Am J Clin Nutr.* 2013;98(2):454-459.

49. Blot WJ, Li JY, Taylor PR, et al. Nutrition intervention trials in Linxian, China: supplementation with specific vitamin/mineral combinations, cancer incidence, and disease-specific mortality in the general population. *J Natl Cancer Inst.* 1993;85(18):1483-1492.

50. Hercberg S, Galan P, Preziosi P, et al. The SU.VI.MAX Study: a randomized, placebo-controlled trial of the health effects of antioxidant vitamins and minerals. *Arch Intern Med.* 2004;164(21):2335-2342.

51. Omenn GS, Goodman GE, Thornquist MD, et al. Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. *N Engl J Med.* 1996;334(18):1150-1155.

52. The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group. *N Engl J Med.* 1994;330(15):1029-1035.

53. Kallio H, Yang B, Peippo P, Tahvonen R, Pan R. Triacylglycerols, glycerophospholipids, tocopherols, and tocotrienols in berries and seeds of two subspecies (ssp. sinensis and mongolica) of Sea Buckthorn (Hippophae rhamnoides). *J Agric Food Chem.* 2002;50(10):3004-3009.

54. Ganju L, Padwad Y, Singh R, et al. Anti-inflammatory activity of Seabuckthorn (Hippophae rhamnoides) leaves. *Int Immunopharmacol.* 2005;5(12):1675-1684.

55. Buyukokuroglu ME, Gulcin I. In vitro antioxidant and antiradical properties of Hippophae rhamnoides L. *Pharmacognosy Magazine.* 2009;5(19):189-195.

56. Geetha S, Sai Ram M, Singh V, Ilavazhagan G, Sawhney RC. Anti-oxidant and immunomodulatory properties of seabuckthorn (Hippophae rhamnoides)--an in vitro study. *J Ethnopharmacol.* 2002;79(3):373-378.

57. Lehtonen HM, Suomela JP, Tahvonen R, et al. Different berries and berry fractions have various but slightly positive effects on the associated variables of metabolic diseases on overweight and obese women. *Eur J Clin Nutr.* 2011;65(3):394-401.

58. Edraki M, Akbarzadeh A, Hosseinzadeh M, Tanideh N, Salehi A, Koohi-Hosseinabadi O. Healing effect of sea buckthorn, olive oil, and their mixture on full-thickness burn wounds. *Adv Skin Wound Care.* 2014;27(7):317-323.

59. Widen C, Renvert S, Persson GR. Antibacterial activity of berry juices, an in vitro study. *Acta Odontol Scand.* 2015:1-5.

60. Johansson AK, Korte H, Yang B, Stanley JC, Kallio HP. Sea buckthorn berry oil inhibits platelet aggregation. *J Nutr Biochem.* 2000;11(10):491-495.

61. Larmo PS, Kangas AJ, Soininen P, et al. Effects of sea buckthorn and bilberry on serum metabolites differ according to baseline metabolic profiles in overweight women: a randomized crossover trial. *Am J Clin Nutr.* 2013;98(4):941-951.

62. O'Callaghan C. *The Renal System at a Glance* 2nd ed. Oxford, Blackwell Publishing Ltd, 2006.

63. Stenvinkel P, Heimburger O, Paultre F, et al. Strong association between malnutrition, inflammation, and atherosclerosis in chronic renal failure. *Kidney Int.*1999;55(5):1899-1911.

64. Foley RN, Parfrey PS, Sarnak MJ. Clinical epidemiology of cardiovascular disease in chronic renal disease. *Am J Kidney Dis.* 1998;32(5 Suppl 3):S112-119.

65. Maisonneuve P, Agodoa L, Gellert R, et al. Cancer in patients on dialysis for end-stage renal disease: an international collaborative study. *Lancet.* 1999;354(9173):93-99.

66. Ghanavatian S, Diep LM, Barany P, et al. Subclinical atherosclerosis, endothelial function, and serum inflammatory markers in chronic kidney disease stages 3 to 4. *Angiology.* 2014;65(5):443-449.

67. Danielski M, Ikizler TA, McMonagle E, et al. Linkage of hypoalbuminemia, inflammation, and oxidative stress in patients receiving maintenance hemodialysis therapy. *Am J Kidney Dis.* 2003;42(2):286-294.

68. Handelman GJ, Walter MF, Adhikarla R, et al. Elevated plasma F2-isoprostanes in patients on long-term hemodialysis. *Kidney Int.* 2001;59(5):1960-1966.

69. Stoyanova E, Sandoval SB, Zuniga LA, et al. Oxidative DNA damage in chronic renal failure patients. *Nephrol Dial Transplant.* 2009;25(3):879-885.

70. Memoli B, Marzano L, Bisesti V, Andreucci M, Guida B. Hemodialysis-related lymphomononuclear release of interleukin-12 in patients with end-stage renal disease. *J Am Soc Nephrol.* 1999;10(10):2171-2176.

71. Lonnemann G. Chronic inflammation in hemodialysis: the role of contaminated dialysate. *Blood Purif.* 2000;18(3):214-223.

72. Gaweda AE, Ginzburg YZ, Chait Y, Germain MJ, Aronoff GR, Rachmilewitz E. Iron dosing in kidney disease: inconsistency of evidence and clinical practice. *Nephrol Dial Transplant.* 2015;30(2):187-196.

73. Ganguli A, Kohli HS, Khullar M, Lal Gupta K, Jha V, Sakhuja V. Lipid peroxidation products formation with various intravenous iron preparations in chronic kidney disease. *Ren Fail.* 2009;31(2):106-110.

74. Agarwal R, Vasavada N, Sachs NG, Chase S. Oxidative stress and renal injury with intravenous iron in patients with chronic kidney disease. *Kidney Int.* 2004;65(6):2279-2289.

75. Capusa C, Mircescu G. Oxidative stress, renal anemia, and its therapies: is there a link? *J Ren Nutr.* 2010;20(5 Suppl):S71-76.

76. National Kidney Foundation. KDOQI Clinical Practice Guidelines and Clinical Practice Recommendations for Anemia in Chronic Kidney Disease. Am J Kidney Dis 2006;47(5 Suppl 3) S16-18.

77. de Mutsert R, Grootendorst DC, Axelsson J, Boeschoten EW, Krediet RT, Dekker FW. Excess mortality due to interaction between protein-energy wasting, inflammation and cardiovascular disease in chronic dialysis patients. *Nephrol Dial Transplant.* 2008;23(9):2957-2964.

78. Leavey SF, Strawderman RL, Jones CA, Port FK, Held PJ. Simple nutritional indicators as independent predictors of mortality in hemodialysis patients. *Am J Kidney Dis.* 1998;31(6):997-1006.

79. Fouque D, Kalantar-Zadeh K, Kopple J, et al. A proposed nomenclature and diagnostic criteria for protein-energy wasting in acute and chronic kidney disease. *Kidney Int.* 2008;73(4):391-398.

80. Fedele S, Sabbah W, Donos N, Porter S, D'Aiuto F. Common oral mucosal diseases, systemic inflammation, and cardiovascular diseases in a large cross-sectional US survey. *Am Heart J.* 2011;161(2):344-350.

81. Ruospo M, Palmer SC, Craig JC, et al. Prevalence and severity of oral disease in adults with chronic kidney disease: a systematic review of observational studies. *Nephrol Dial Transplant.* 2014;29(2):364-375.

82. Tomas I, Marinho JS, Limeres J, Santos MJ, Araujo L, Diz P. Changes in salivary composition in patients with renal failure. *Arch Oral Biol.* 2008;53(6):528-532.

83. Thorman R, Neovius M, Hylander B. Clinical findings in oral health during progression of chronic kidney disease to end-stage renal disease in a Swedish population. *Scand J Urol Nephrol.* 2009;43(2):154-159.

84. Gavalda C, Bagan J, Scully C, Silvestre F, Milian M, Jimenez Y. Renal hemodialysis patients: oral, salivary, dental and periodontal findings in 105 adult cases. *Oral Dis.*1999;5(4):299-302.

85. Proctor R, Kumar N, Stein A, Moles D, Porter S. Oral and dental aspects of chronic renal failure. *J Dent Res.* 2005;84(3):199-208.

86. Mashayekhi F, Aghahoseini F, Rezaie A, Zamani MJ, Khorasani R, Abdollahi M. Alteration of cyclic nucleotides levels and oxidative stress in saliva of human subjects with periodontitis. *J Contemp Dent Pract.* 2005;6(4):46-53.

87. Roduner E. Size matters: why nanomaterials are different. *Chem Soc Rev.* 2006;35(7):583-592.

88. Auffan M, Rose J, Bottero JY, Lowry GV, Jolivet JP, Wiesner MR. Towards a definition of inorganic nanoparticles from an environmental, health and safety perspective. *Nat Nanotechnol.* 2009;4(10):634-641.

89. Buzea C, Pacheco, II, Robbie K. Nanomaterials and nanoparticles: sources and toxicity. *Biointerphases.* 2007;2(4):MR17-71.

90. Nanotechnology and human health: Scientific evidence and risk governance. Report of the WHO expert meeting 10–11 December 2012, Bonn, Germany. Copenhagen, WHO Regional Office for Europe, 2013.

91. SCENIHR (Scientific Committee on Emerging and Newly-Identified Health Risks). The appropriateness of the risk assessment methodology in accordance with the Technical Guidance Documents for new and existing substances for assessing the risks of nanomaterials, 21-22 June 2007.

92. European commission. Commission recommendation of 18 October 2011 on the definition of nanomaterial. *Official Journal of the European Union.* 2011.

93. Roco MC, Mirkin CA, Hersam MC. WTEC Panel Report on Nanotechnology Research Directions for Societal Needs in 2020 Retrospective and Outlook. *Springer series: Science policy reports, 2011.*

94. The project of emerging nanotechnologies, A nanotechnology consumer product inventory. Available at http://www.nanotechproject.org [Cited 2015-02].

95. Chaloupka K, Malam Y, Seifalian AM. Nanosilver as a new generation of nanoproduct in biomedical applications. *Trends Biotechnol.* 2010;28(11):580-588.

96. Weir A, Westerhoff P, Fabricius L, Hristovski K, von Goetz N. Titanium dioxide nanoparticles in food and personal care products. *Environ Sci Technol.* 2012;46(4):2242-2250.

97. Evans P, Matsunaga H, Kiguchi M. Large-scale application of nanotechnology for wood protection. *Nat Nanotechnol.* 2008;3(10):577.

98. Kyzas G., Matis KA. Nanoadsorbents for pollutants removal: A review. *Journal of Molecular Liquids.* 2015;203:159-168.

99. Zeleňáka V, Badaničováa M, Halamováa D, et al. Amine-modified ordered mesoporous silica: Effect of pore size on carbon dioxide capture. *Chemical Engineering Journal.* 2008;144(2):336-342.

100. Wicki A, Witzigmann D, Balasubramanian V, Huwyler J. Nanomedicine in cancer therapy: challenges, opportunities, and clinical applications. *J Control Release.* 2015;200:138-157.

101. Rizzo LY, Theek B, Storm G, Kiessling F, Lammers T. Recent progress in nanomedicine: therapeutic, diagnostic and theranostic applications. *Curr Opin Biotechnol.* 2013;24(6):1159-1166.

102. Nehoff H, Parayath NN, Domanovitch L, Taurin S, Greish K. Nanomedicine for drug targeting: strategies beyond the enhanced permeability and retention effect. *Int J Nanomedicine.* 2014;9:2539-2555.

103. Pissuwan D, Niidome T. Polyelectrolyte-coated gold nanorods and their biomedical applications. *Nanoscale.* 2015;7(1):59-65.

104. Gao L, Nie L, Wang T, et al. Carbon nanotube delivery of the GFP gene into mammalian cells. *Chembiochem.* 2006;7(2):239-242.

105. Liu H, Wang Y, Wang M, Xiao J, Cheng Y. Fluorinated poly(propylenimine) dendrimers as gene vectors. *Biomaterials.* 2014;35(20):5407-5413.

106. Brevet D, Hocine O, Delalande A, et al. Improved gene transfer with histidine-functionalized mesoporous silica nanoparticles. *Int J Pharm.* 2014;471(1-2):197-205.

107. Wang T, Upponi JR, Torchilin VP. Design of multifunctional non-viral gene vectors to overcome physiological barriers: dilemmas and strategies. *Int J Pharm.* 2012;427(1):3-20.

108. Argyo C, Weiss V, Bräuchle C, Bein T. Multifunctional Mesoporous Silica Nanoparticles as a Universal Platform for Drug Delivery. *Chem. of Mater.* 2014;26(1):435-451.

109. Gref R, Luck M, Quellec P, et al. 'Stealth' corona-core nanoparticles surface modified by polyethylene glycol (PEG): influences of the corona (PEG chain length and surface density) and of the core composition on phagocytic uptake and plasma protein adsorption. *Colloids Surf B Biointerfaces.* 2000;18(3-4):301-313.

110. Niedermayer S, Weiss V, Herrmann A, et al. Multifunctional polymer-capped mesoporous silica nanoparticles for pH-responsive targeted drug delivery. *Nanoscale.* 2015;7(17):7953-64.

111. Gimenez C, de la Torre C, Gorbe M, et al. Gated mesoporous silica nanoparticles for the controlled delivery of drugs in cancer cells. *Langmuir.* 2015;31(12):3753-3762.

112. Donaldson K, Stone V, Tran CL, Kreyling W, Borm PJ. Nanotoxicology. *Occup Environ Med.* 2004;61(9):727-728.

113. Donaldson K, Poland CA. Nanotoxicity: challenging the myth of nano-specific toxicity. *Curr Opin Biotechnol.* 2013;24(4):724-734.

114. Hassing C, Twickler M, Brunekreef B, et al. Particulate air pollution, coronary heart disease and individual risk assessment: a general overview. *Eur J Cardiovasc Prev Rehabil.* 2009;16(1):10-15.

115. ICRP. Human Respiratory Tract Model for Radiological Protection. ICRP Publication 66. Ann. ICRP 24 (1-3). 1994.

116. Geiser M, Kreyling WG. Deposition and biokinetics of inhaled nanoparticles. *Part Fibre Toxicol.* 2010;7:2.

117. Moller W, Felten K, Sommerer K, et al. Deposition, retention, and translocation of ultrafine particles from the central airways and lung periphery. *Am J Respir Crit Care Med.* 2008;177(4):426-432.

118. Geiser M, Casaulta M, Kupferschmid B, Schulz H, Semmler-Behnke M, Kreyling W. The role of macrophages in the clearance of inhaled ultrafine titanium dioxide particles. *Am J Respir Cell Mol Biol.* 2008;38(3):371-376.

119. Donaldson K, Poland CA, Murphy FA, MacFarlane M, Chernova T, Schinwald A. Pulmonary toxicity of carbon nanotubes and asbestos - similarities and differences. *Adv Drug Deliv Rev.* 2013;65(15):2078-2086.

120. Nemmar A, Hoet PH, Vanquickenborne B, et al. Passage of inhaled particles into the blood circulation in humans. *Circulation.* 2002;105(4):411-414.

121. Brown JS, Zeman KL, Bennett WD. Ultrafine particle deposition and clearance in the healthy and obstructed lung. *Am J Respir Crit Care Med.* 2002;166(9):1240-1247.

122. Mills NL, Amin N, Robinson SD, et al. Do inhaled carbon nanoparticles translocate directly into the circulation in humans? *Am J Respir Crit Care Med.* 2006;173(4):426-431.

123. Wiebert P, Sanchez-Crespo A, Falk R, et al. No significant translocation of inhaled 35-nm carbon particles to the circulation in humans. *Inhal Toxicol.* 2006;18(10):741-747.

124. Powell JJ, Faria N, Thomas-McKay E, Pele LC. Origin and fate of dietary nanoparticles and microparticles in the gastrointestinal tract. *J Autoimmun.* 2010;34(3):J226-233.

125. Hillyer JF, Albrecht RM. Gastrointestinal persorption and tissue distribution of differently sized colloidal gold nanoparticles. *J Pharm Sci.* 2001;90(12):1927-1936.

126. Hoet PH, Bruske-Hohlfeld I, Salata OV. Nanoparticles - known and unknown health risks. *J Nanobiotechnology.* 2004;2(1):12.

127. Filipe P, Silva JN, Silva R, et al. Stratum corneum is an effective barrier to TiO2 and ZnO nanoparticle percutaneous absorption. *Skin Pharmacol Physiol.* 2009;22(5):266-275.

128. Watkinson AC, Bunge AL, Hadgraft J, Lane ME. Nanoparticles do not penetrate human skin--a theoretical perspective. *Pharm Res.* 2013;30(8):1943-1946.

129. Labouta HI, Schneider M. Interaction of inorganic nanoparticles with the skin barrier: current status and critical review. *Nanomedicine.* 2013;9(1):39-54.

130. Manke A, Wang L, Rojanasakul Y. Mechanisms of nanoparticle-induced oxidative stress and toxicity. *Biomed Res Int.* 2013;2013:942916.

131. Murugan K, Choonara YE, Kumar P, Bijukumar D, du Toit LC, Pillay V. Parameters and characteristics governing cellular internalization and trans-barrier trafficking of nanostructures. *Int J Nanomedicine.* 2015;10:2191-2206.

132. Zhao F, Zhao Y, Liu Y, Chang X, Chen C, Zhao Y. Cellular uptake, intracellular trafficking, and cytotoxicity of nanomaterials. *Small.* 2011;7(10):1322-1337.

133. Li N, Xia T, Nel AE. The role of oxidative stress in ambient particulate matter-induced lung diseases and its implications in the toxicity of engineered nanoparticles. *Free Radic Biol Med.* 2008;44(9):1689-1699.

134. Sinani D, Adle DJ, Kim H, Lee J. Distinct mechanisms for Ctr1-mediated copper and cisplatin transport. *J Biol Chem.* 2007;282(37):26775-26785.

135. Studer AM, Limbach LK, Van Duc L, et al. Nanoparticle cytotoxicity depends on intracellular solubility: comparison of stabilized copper metal and degradable copper oxide nanoparticles. *Toxicol Lett.* 2010;197(3):169-174.

136. Cronholm P, Karlsson HL, Hedberg J, et al. Intracellular uptake and toxicity of Ag and CuO nanoparticles: a comparison between nanoparticles and their corresponding metal ions. *Small.* 2013;9(7):970-982.

137. Chen S, Hou Y, Cheng G, Zhang C, Wang S, Zhang J. Cerium oxide nanoparticles protect endothelial cells from apoptosis induced by oxidative stress. *Biol Trace Elem Res.* 2013;154(1):156-166.

138. Korsvik C, Patil S, Seal S, Self WT. Superoxide dismutase mimetic properties exhibited by vacancy engineered ceria nanoparticles. *Chem Commun (Camb).* 2007(10):1056-1058.

139. Magdolenova Z, Collins A, Kumar A, Dhawan A, Stone V, Dusinska M. Mechanisms of genotoxicity. A review of in vitro and in vivo studies with engineered nanoparticles. *Nanotoxicology.* 2014;8(3):233-278.

140. Huang K, Ma H, Liu J, et al. Size-dependent localization and penetration of ultrasmall gold nanoparticles in cancer cells, multicellular spheroids, and tumors in vivo. *ACS Nano.* 2012;6(5):4483-4493.

141. Fan J1 LH, Jiang J, So LK, Lam YW, Chu PK. 3C–SiC Nanocrystals as Fluorescent Biological labels. *Small.* 2008;4(8):1058-1062

142. Nabeshi H, Yoshikawa T, Matsuyama K, et al. Systemic distribution, nuclear entry and cytotoxicity of amorphous nanosilica following topical application. *Biomaterials.* 2011;32(11):2713-2724.

143. Stoccoro A, Karlsson HL, Coppede F, Migliore L. Epigenetic effects of nano-sized materials. *Toxicology.* 2013;313(1):3-14.

144. Kagan VE, Bayir H, Shvedova AA. Nanomedicine and nanotoxicology: two sides of the same coin. *Nanomedicine.* 2005;1(4):313-316.

145. Konduru NV, Tyurina YY, Feng W, et al. Phosphatidylserine targets single-walled carbon nanotubes to professional phagocytes in vitro and in vivo. *PLoS One.* 2009;4(2):e4398.

146. Ilinskaya AN, Dobrovolskaia MA. Immunosuppressive and anti-inflammatory properties of engineered nanomaterials. *Br J Pharmacol.* 2014;171(17):3988-4000.

147. Lundborg M, Johard U, Lastbom L, Gerde P, Camner P. Human alveolar macrophage phagocytic function is impaired by aggregates of ultrafine carbon particles. *Environ Res.* 2001;86(3):244-253.

148. Tenzer S, Docter D, Kuharev J, et al. Rapid formation of plasma protein corona critically affects nanoparticle pathophysiology. *Nat Nanotechnol.* 2013;8(10):772-781.

149. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res.* 1988;175(1):184-191.

150. Hamm ML, Gill TJ, Nicolson SC, Summers MR. Substrate specificity of Fpg (MutM) and hOGG1, two repair glycosylases. *J Am Chem Soc.* 2007;129(25):7724-7725.

151. Borawski J, Wilczynska-Borawska M, Stokowska W, Mysliwiec M. The periodontal status of pre-dialysis chronic kidney disease and maintenance dialysis patients. *Nephrol Dial Transplant.* 2007;22(2):457-464.

152. Stopper H, Boullay F, Heidland A, Vienken J, Bahner U. Comet-assay analysis identifies genomic damage in lymphocytes of uremic patients. *Am J Kidney Dis.* 2001;38(2):296-301.

153. Sandoval SB, Stoyanova E, Coll E, et al. Genetic damage in chronic renal failure patients is associated with the glomerular filtration rate index. *Mutagenesis.* 2010;25(6):603-608.

154. Corredor Z, Stoyanova E, Rodriguez-Ribera L, et al. Genomic damage as a biomarker of chronic kidney disease status. *Environ Mol Mutagen.* 2015;56(3):301-312.

155. Schupp N, Stopper H, Rutkowski P, et al. Effect of different hemodialysis regimens on genomic damage in end-stage renal failure. *Semin Nephrol.* 2006;26(1):28-32.

156. Herman M, Ori Y, Chagnac A, et al. Spontaneous DNA repair increases during hemodialysis. *Nephron Clin Pract.* 2008;108(3):c188-193.

157. Bibi G, Green Y, Nagler RM. Compositional and oxidative analysis in the saliva and serum of predialysis chronic kidney disease patients and end-stage renal failure patients on peritoneal dialysis. *Ther Apher Dial.* 2008;12(2):164-170.

158. Kho HS, Lee SW, Chung SC, Kim YK. Oral manifestations and salivary flow rate, pH, and buffer capacity in patients with end-stage renal disease undergoing hemodialysis. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.* 1999;88(3):316-319.

159. Narayanan S, Ruma D, Gitika B, et al. Antioxidant activities of seabuckthorn (Hippophae rhamnoides) during hypoxia induced oxidative stress in glial cells. *Mol Cell Biochem.* 2005;278(1-2):9-14.

160. Shukla SK, Chaudhary P, Kumar IP, et al. Protection from radiation-induced mitochondrial and genomic DNA damage by an extract of Hippophae rhamnoides. *Environ Mol Mutagen.* 2006;47(9):647-656.

161. Salem S, Bruck H, Bahlmann FH, et al. Relationship between Magnesium and Clinical Biomarkers on Inhibition of Vascular Calcification. *Am J Nephrol.* 2012;35(1):31-39.

162. Rivara MB, Mehrotra R, Linke L, Ruzinski J, Ikizler TA, Himmelfarb J. A pilot randomized crossover trial assessing the safety and short-term effects of pomegranate supplementation in hemodialysis patients. *J Ren Nutr.* 2015;25(1):40-49.

163. Shema-Didi L, Sela S, Ore L, et al. One year of pomegranate juice intake decreases oxidative stress, inflammation, and incidence of infections in hemodialysis patients: a randomized placebo-controlled trial. *Free Radic Biol Med.* 2012;53(2):297-304.

164. Eccleston C, Baoru Y, Tahvonen R, Kallio H, Rimbach GH, Minihane AM. Effects of an antioxidant-rich juice (sea buckthorn) on risk factors for coronary heart disease in humans. *J Nutr Biochem.* 2002;13(6):346-354.

165. Halliwell B. Cell culture, oxidative stress, and antioxidants: avoiding pitfalls. *Biomed J.* 2014;37(3):99-105.

166. Liu G, Gao J, Ai H, Chen X. Applications and potential toxicity of magnetic iron oxide nanoparticles. *Small.* 2013;9(9-10):1533-1545.

167. Srinivas A, Rao PJ, Selvam G, Goparaju A, Murthy PB, Reddy PN. Oxidative stress and inflammatory responses of rat following acute inhalation exposure to iron oxide nanoparticles. *Hum Exp Toxicol.* 2012;31(11):1113-1131.

168. Napierska D, Thomassen LC, Lison D, Martens JA, Hoet PH. The nanosilica hazard: another variable entity. *Part Fibre Toxicol.* 2010;7(1):39.

169. Semisch A, Ohle J, Witt B, Hartwig A. Cytotoxicity and genotoxicity of nano - and microparticulate copper oxide: role of solubility and intracellular bioavailability. *Part Fibre Toxicol.* 2014;11(1):10.

170. Karlsson HL, Gustafsson J, Cronholm P, Möller L. Size-dependent toxicity of metal oxide particles--a comparison between nano- and micrometer size. *Toxicol Lett.* 2009;188(2):112-118.

171. Bartlomiejczyk T, Lankoff A, Kruszewski M, Szumiel I. Silver nanoparticles -- allies or adversaries? *Ann Agric Environ Med.* 2013;20(1):48-54.

172. Bregoli L, Chiarini F, Gambarelli A, et al. Toxicity of antimony trioxide nanoparticles on human hematopoietic progenitor cells and comparison to cell lines. *Toxicology.* 2009;262(2):121-129.

173. Lanone S, Rogerieux F, Geys J, et al. Comparative toxicity of 24 manufactured nanoparticles in human alveolar epithelial and macrophage cell lines. *Part Fibre Toxicol.* 2009;6:14.

174. Chang JS, Chang KL, Hwang DF, Kong ZL. In vitro cytotoxicitiy of silica nanoparticles at high concentrations strongly depends on the metabolic activity type of the cell line. *Environ Sci Technol.* 2007;41(6):2064-2068.

175. Stohs SJ, Bagchi D. Oxidative mechanisms in the toxicity of metal ions. *Free Radic Biol Med.* 1995;18(2):321-336.

176. Karlsson HL, Cronholm P, Hedberg Y, et al. Cell membrane damage and protein interaction induced by copper containing nanoparticles--importance of the metal release process. *Toxicology.* 2013;313(1):59-69.

177. Ivanov AI. Exocytosis and endocytosis. Preface. *Methods Mol Biol.* 2008;440:v-vi.

178. Siddiqui MA, Alhadlaq HA, Ahmad J, Al-Khedhairy AA, Musarrat J, Ahamed M. Copper oxide nanoparticles induced mitochondria mediated apoptosis in human hepatocarcinoma cells. *PLoS One.* 2013;8(8):e69534.

179. Midander K, Cronholm P, Karlsson HL, et al. Surface characteristics, copper release, and toxicity of nano- and micrometer-sized copper and copper(II) oxide particles: a cross-disciplinary study. *Small.* 2009;5(3):389-399.

180. Li F, Lei C, Shen Q, et al. Analysis of copper nanoparticles toxicity based on a stress-responsive bacterial biosensor array. *Nanoscale.* 2013;5(2):653-662.

181. Vanwinkle BA, de Mesy Bentley KL, Malecki JM, et al. Nanoparticle (NP) uptake by type I alveolar epithelial cells and their oxidant stress response. *Nanotoxicology.* 2009;3(4):307-318.

182. Menkissoglu O, Lindow SE. Relationships of free ionic copper and toxicity to bacteria in solutions of organic-compounds. *Phytopathology.* 1991;81(10):1258-1263.

183. Cronholm P, Midander K, Karlsson HL, Elihn K, Odnevall Wallinder I, Möller L. Effect of sonication and serum proteins on copper release from

copper nanoparticles and the toxicity towards lung epithelial cells. *Nanotoxicology.* 2011;5(2):269-281.

184. Kim JA, Salvati A, Aberg C, Dawson KA. Suppression of nanoparticle cytotoxicity approaching in vivo serum concentrations: limitations of in vitro testing for nanosafety. *Nanoscale.* 2014;6(23):14180-14184.

185. Holder AL, Goth-Goldstein R, Lucas D, Koshland CP. Particle-Induced Artifacts in the MTT and LDH Viability Assays. *Chem Res Toxicol.* 2012;25(9):1885-1892.

186. Kroll A, Pillukat MH, Hahn D, Schnekenburger J. Interference of engineered nanoparticles with in vitro toxicity assays. *Arch Toxicol.* 2012;86(7):1123-1136.

187. Galluzzi L, Vitale I, Abrams JM, et al. Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. *Cell Death Differ.* 2012;19(1):107-120.

188. Collins AR, Oscoz AA, Brunborg G, et al. The comet assay: topical issues. *Mutagenesis.* 2008;23(3):143-151.

189. Singh N, Karambelkar A, Gu L, et al. Bioresponsive mesoporous silica nanoparticles for triggered drug release. *J Am Chem Soc.* 2011;133(49):19582-19585.

190. Zhu M, Nie G, Meng H, Xia T, Nel A, Zhao Y. Physicochemical properties determine nanomaterial cellular uptake, transport, and fate. *Acc Chem Res.* 2013;46(3):622-631.

191. Frohlich E. The role of surface charge in cellular uptake and cytotoxicity of medical nanoparticles. *Int J Nanomedicine.* 2012;7:5577-5591.

192. Li SD, Huang L. Pharmacokinetics and biodistribution of nanoparticles. *Mol Pharm.* 2008;5(4):496-504.

193. Chen Y, Chen H, Shi J. In vivo bio-safety evaluations and diagnostic/therapeutic applications of chemically designed mesoporous silica nanoparticles. *Adv Mater.* 2013;25(23):3144-3176.

194. Kim MH, Na HK, Kim YK, et al. Facile synthesis of monodispersed mesoporous silica nanoparticles with ultralarge pores and their application in gene delivery. *ACS Nano.* 2011;5(5):3568-3576.

195. Na HK, Kim MH, Park K, et al. Efficient functional delivery of siRNA using mesoporous silica nanoparticles with ultralarge pores. *Small.* 2012;8(11):1752-1761.

196. Slowing, II, Wu CW, Vivero-Escoto JL, Lin VS. Mesoporous silica nanoparticles for reducing hemolytic activity towards mammalian red blood cells. *Small.* 2009;5(1):57-62.

197. Xia T, Kovochich M, Liong M, et al. Polyethyleneimine coating enhances the cellular uptake of mesoporous silica nanoparticles and allows safe delivery of siRNA and DNA constructs. *ACS Nano.* 2009;3(10):3273-3286.

198. Ye SF, Tian MM, Wang TX, et al. Synergistic effects of cell-penetrating peptide Tat and fusogenic peptide HA2-enhanced cellular internalization and gene transduction of organosilica nanoparticles. *Nanomedicine.* 2012;8(6):833-841.

199. Shi J, Karlsson HL, Johansson K, et al. Microsomal glutathione transferase 1 protects against toxicity induced by silica nanoparticles but not by zinc oxide nanoparticles. *ACS Nano.* 2012;6(3):1925-1938.

200. Cedervall T, Lynch I, Lindman S, et al. Understanding the nanoparticle-protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles. *Proc Natl Acad Sci U S A.* 2007;104(7):2050-2055.

201. Shi J, Hedberg Y, Lundin M, Odnevall Wallinder I, Karlsson HL, Moller L. Hemolytic properties of synthetic nano- and porous silica particles: the effect of surface properties and the protection by the plasma corona. *Acta Biomater.* 2012;8(9):3478-3490.

202. Cronholm P, Midander K, Karlsson HL, Elihn K, Wallinder IO, Moller L. Effect of sonication and serum proteins on copper release from copper nanoparticles and the toxicity towards lung epithelial cells. *Nanotoxicology.* 2011;5(2):269-281.